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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Oxford BioMedica (UK) Limited,
Medawar Centre,
Oxford Science Park,
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Patents ADP number *(if you know it)*

7667157001

If the applicant is a corporate body, give the country/state of its incorporation

United Kingdom

4. Title of the invention

5T4 ANTIGEN EXPRESSION

5. Name of your agent *(if you have one)*

D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

21 New Fetter Lane
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EC4A 1DA

Patents ADP number *(if you know it)*

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Signature D Young & Co Date 02 July 2002

D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom: Antonio Maschio 023 8071 9500

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5T4 antigen expression

Field of the Invention

The present invention relates to the identification that expression of 5T4 antigen is switched on during stem cell differentiation. Accordingly, detection of 5T4 expression can be used as an indicator of the differentiation status of stem cells.

Background to the Invention

Mammalian stem cells are undifferentiated, primitive cells which have the ability both to multiply and to differentiate into specific kinds of cells. Embryos provide a high concentration of stem cells and stem cell lines derived from embryos, embryonic stem (ES) cells, are pluripotent, thus possessing the capability of developing into any cell. These cells are immortal and can be maintained in an undifferentiated state in culture or directed to undergo differentiation into extraembryonic or somatic lineages. More recently, it has been recognised that embryonic germ (EG) cells i.e. cells derived from primordial germ cells may have similar properties to ES cells. Other stem cells may be derived from adults and include mesenchymal, epithelial and neural stem cells.

Such stem cells represent a major potential for cell therapies for regenerative medicine as differentiated cells can be generated for transplantation, may be genetically modified and can be transplanted as pure populations or, following tissue engineering, as tissues or physiologically functional parts of organs (organoids). ES cells are also useful models for studying the cellular and molecular biology of early development and functional genomics. *In vitro* culture of stem cells can also provide a useful system for drug screening and drug discovery. ES cells derived from mouse embryos are routinely used in a number of laboratory techniques ranging from gene knockout studies, for example generating "knock out" mice models, to transplantation therapies (Sato et al. (2001)).

Stem cells are generally difficult to culture *in vitro* and careful control of culture conditions, including the appropriate quality of serum and culture medium, is required. This is particularly important if such cells are to be genetically modified or manipulated to introduce genetic mutations, to be grown on a large scale or to direct their differentiation towards

specific cell types. In addition, careful control and analysis of the differentiation status is required to ensure that the cultured stem cells are suited for their particular use. The selection of appropriate starting cells for directing appropriate phenotypic differentiation is essential as failure can lead not only to a lack of benefit but also to significant side-effects which can include proliferation of undifferentiated cells. In particular, if cells are not fully differentiated at the time of implantation there is always the possibility of tumour formation. It is therefore clearly important to be able to confirm and select for the undifferentiated integrity or differentiation state of cells within a stem cell population.

Some markers of the status of stem cells are known. Markers currently used for analysis of the undifferentiated integrity of ES cells include Oct 3/4 (Rathjen et al. (1999)), Rex-1 (Ben-Sushan et al. (1998)), the cell-surface Forssman antigen (Willison et al. (1978); Ling et al. (1997)) and alkaline phosphatase (Rathjen et al. (1999)) (Table 1). All these markers are expressed in undifferentiated ES cells and their levels decrease upon differentiation. However, they are not useful for predicting both the undifferentiated integrity and differentiation state of ES cells since they decrease relatively slowly following the onset of differentiation (Lake et al. (2000); Rathjen et al. (1999)). Additionally, with the exception of the Forssman antigen, the analyses are destructive to cells and require relatively large numbers of cells for RNA extraction.

Removal of leukaemia inhibitory factor (LIF) from the medium results in ES cell differentiation (Smith et al. (1992)), characterised by the upregulation of transcript markers such as fibroblast growth factor-5 (Fgf-5), zeta globin (ZG) and Flk-1 (Table 1). However, these markers are transiently expressed and present only on a sub-population of cells thereby limiting their use as single assay markers of ES cell integrity and differentiation.

To date, there is no marker that can accurately assess both the undifferentiated integrity and differentiated state of stem cells. Current analyses of these parameters are time-consuming, often destructive to cells, and require several different markers (Weinhold et al. (2000); Lake et al (2000); Rathjen et al. (1999)). Analysis in a single, non-destructive assay would be a valuable tool for a wide range of ES cell techniques (Lake et al. (2000); Thorey et al. (1998); Niwa et al. (2000); Wakayama et al (1999)).

The 5T4 oncofoetal antigen is a 72 kDa highly glycosylated single pass transmembrane glycoprotein originally isolated from human placental trophoblast. (Hole, N. & Stern, P. L. (1988); Hole, N. & Stern, P. L. (1990) and Myers, K. A. et al. (1994). 5T4 has been extensively characterised (see, for example, WO 89/07947). It exhibits restricted expression patterns in human adult tissues, being expressed by trophoblast and a few specialised adult epithelia, but is upregulated on many carcinomas, with tumour overexpression correlating with poorer clinical outcome in ovarian, gastric and colorectal cancers. (Southall, P. J. et al. (1990); Wrigley, E. et al. (1995); Starzynska, T. et al. (1994); Starzynska, T. et al. (1998); Mulder, W. M. et al. (1997); Starzynska, T. et al. (1992)). The pattern of 5T4 expression in stem cell populations has not previously been identified.

Summary of the Invention

The present application identifies that the expression of 5T4-oncofoetal antigen is a marker of differentiated ES cells. 5T4 protein and mRNA are not detectable in undifferentiated ES cells but are rapidly upregulated in cells derived from all three germ layers following differentiation. Thus, we demonstrate that lack of cell-surface 5T4 antigen is a sensitive indicator of undifferentiated ES cell integrity, allowing rapid monitoring and optimising of ES cell culture conditions. 5T4 antigen expression on ES cells is unaffected by extended passage, cloning or growth on gelatin-treated plates, allowing differentiation analysis for a wide range of ES cell appplications. By contrast, ES cell transcript markers Oct-3/4 or Rex-1 (Rathjen et al. (1999); Niwa et al. (2000); Ben-Sushan et al. (1998)) are unable to confirm homogeneous ES cell integrity since they continue to be expressed in differentiating 5T4-positive monolayer cultures.

Accordingly, in one aspect of the invention, there is provided a method for detecting the differentiation status of stem cells comprising detecting expression of 5T4 antigen wherein lack of expression of 5T4 indicates undifferentiated stem cells whereas an increased level of expression indicates stem cells which have activated the differentiation pathway. Preferably, said stem cells are mammalian stem cells and, in particular, ES cells.

Expression of 5T4 antigen can be detected through detection of mRNA transcripts or through detection of the 5T4 protein. Techniques for detecting gene and protein expression are familiar to those skilled in the art.

As demonstrated herein, the level of 5T4 expression correlates with the differentiation status of the stem cells such as ES cells. Thus, an absence or lack of 5T4 expression is no 5T4 expression or a low or negligible level of 5T4 expression and indicates that the stem cells are undifferentiated whereas an increased amount of expression compared to this low level indicates the presence of differentiated cells. Suitably the level of 5T4 expression may be determined through comparative studies of stem cells incubated under different conditions. Levels may be expressed as numbers or % of positive cells in a stem cell population when measured by FACS based techniques or through quantitative analysis methods such as quantitative amplification of mRNAs (e.g. RT-PCR) or quantitative determination of protein expression (e.g. Western Blotting). Suitable methods are described herein.

In a preferred embodiment there is provided a method of detecting differentiation status of mammalian stem cells comprising the steps of:

a) taking a sample of stem cells;
b) incubating said sample with a labelled anti-5T4 antibody such that specific binding of anti-5T4 antibody to 5T4 antigen occurs;
c) detecting said binding.

Suitably, the method for detecting 5T4 expression is an immunofluorescent technique in which fluorescently labelled anti-5T4 antibody is used and detection is through FACS analysis substantially as described herein. In this embodiment, it is preferred that the anti-5T4 antibody specifically recognises an extracellularly expressed portion of 5T4. The detection of 5T4 antibody or 5T4 tagged antibody by anti-Ig or anti-tag Abs are envisaged.

Suitably, said mammalian stem cells are derived from embryos and include embryonic stem cells (ES cells), embryonic germ cells or embryonal carcinoma cells. Other suitable cells are adult stem cells and include mesenchymal, haematopoeitic, neural and epithelial cells. In one embodiment, said cells are genetically modified stem cells.

Said stem cells are suitably murine, human, porcine, feline or canine although any mammalian stem cells may be used.

In another aspect there is provided use of anti-5T4 antibodies in a method for detecting differentiation status of mammalian stem cells.

Suitable anti-5T4 antibodies include those known in the art or any anti-5T4 antibodies that can be raised according to methods known to those skilled in the art. In one embodiment, the anti-5T4 antibody is the 9A7 antibody as described herein. Preferably, the anti-5T4 antibody recognises the extracellular domain of the 5T4 antigen to facilitate detection of 5T4 cell surface expression and thus allow non-destructive detection methods. Methods for labelling antibodies to detect binding are known to those skilled in the art.

Cultured mammalian stem cells can be used in a number of techniques. In some techniques it is desirable to use a population of cells comprising only differentiated or only undifferentiated cells.

Accordingly, in another aspect of the invention, there is provided a method for separating a population of undifferentiated or differentiated mammalian stem cells from a mixture of differentiated and undifferentiated stem cells comprising:

a) binding cells with anti-5T4 antibody;
b) separating cells with bound antibody from cells with no bound antibody;
c) unbinding the antibody from the cells; and
d) isolating the cells.

Suitable methods for separating cells include using Ig magnetic beads such as MACS beads or other FACS techniques. It will be appreciated that where a population of undifferentiated stem cells is desired, those cells with no bound antibody may be isolated and selected.

In a preferred embodiment, the cells isolated or separated by said method are viable.

In a further aspect of the invention there is provided a method for testing growth serum for its use in maintaining mammalian cells comprising detecting expression of 5T4.

Suitably, said method comprises the steps of:

a) taking mammalian stem cells in culture;

b) applying test media; and

c) assessing 5T4 expression in the absence or presence of said media wherein the presence of 5T4 is an indication that mammalian stem cells are undergoing differentiation.

Stem cells represent useful culture conditions for detecting effects of a test compound and in particular detecting the ability of a test compound to induce differentiation or cause any toxic effects.

Accordingly, in a further aspect of the invention, there is provided a method for detecting the ability of a test compound to induce mammalian stem cell differentiation comprising the steps of:

a) incubating a mammalian cell culture in the presence or absence of said test compound;

b) detecting 5T4 expression; and

c) comparing the levels of 5T4 expression in cells wherein increased 5T4 expression in those cells incubated in the presence of said test compound indicates differentiation induction by said test compound.

In one embodiment, "5T4 expression" may be detected through detecting 5T4 promoter activity in a construct in which the 5T4 promoter is operably linked to a reporter gene as described below.

The detection of 5T4 mRNA and protein expression at the beginning of stem cell differentiation suggests that activation of 5T4 transcription may be a key event in the induction of differentiation and developmental pathways.

Thus, the detection of 5T4 expression can be an indication of the induction of differentiation by a known compound. Suitable differentiation-inducing compounds are known to those skilled in the art. Thus, the ability of a test compound to act as an enhancer or inhibitor of the activity of a differentiation-inducing compound can be detected by measuring 5T4 expression.

Accordingly, in another aspect of the invention, there is provided a method for detecting the ability of a test compound to enhance or inhibit the activity of a mammalian stem cell differentiation-inducing compound comprising the steps of:

a) incubating a mammalian cell culture treated with a differentiation-inducing compound in the presence or absence of said test compound;
b) detecting 5T4 expression; and
c) comparing the levels of 5T4 expression in cells wherein increased 5T4 expression in those cells incubated in the presence of said test compound indicates the ability of a test compound to enhance differentiation-induction while decreased 5T4 expression indicates the ability of a test compound to inhibit differentiation-induction.

Transcription of 5T4 may be regulated by interactions at the level of promoter activation from the 5T4 gene promoter region. Activation of the 5T4 promoter may be harnessed to induce expression of genes at the beginning of the stem cell differentiation pathway. Suitable genes which may be expressed under the control of the 5T4 promoter include those which may act as reporter genes to allow expression of selectable markers or expression of genes conferring resistance to selectable conditions such as neomycin. Other suitable genes include functional genes for which expression at the beginning of differentiation may be desirable such as genes involved in specific differentiation pathways. In addition, it may be desirable to express to genes whose products have a toxic effect on a cell. In this way, expression of the gene under control of the 5T4 promoter would induce expression of a toxic product in those cells undergoing differentiation and therefore eradicate differentiating cells from a population.

Accordingly in another aspect of the invention there is provided a method for detecting differentiation status of a mammalian stem cell comprising:

a) introducing into a stem cell a vector comprising a 5T4 promoter sequence operably linked to a nucleic acid encoding a reporter gene; and
b) detecting an increase in expression of the reporter gene as an indication of differentiation.

In a further aspect of the invention, there is provided a method of modifying a mammalian stem cell comprising introducing a nucleic acid sequence into a mammalian cell such that said nucleic acid sequence is placed under the control of the 5T4 promoter sequence.

In one embodiment, genes may be expressed under the control of the 5T4 promoter region through introduction of vectors comprising the 5T4 promoter operably linked to the nucleic acid encoding the gene of interest. In another embodiment, the genes introduced may be combined or "knocked in" to the genome of the stem cell through methods such as homologous recombination. Other suitable methods will be familiar to those skilled in the art.

In another aspect of the invention, there is provided a method of modulating stem cell differentiation comprising modifying the expression of 5T4 or its functional activity.

Cells which have been sorted according to their expression of 5T4 can be used in a number of stem cell applications. Accordingly, in another aspect of the invention, there is provided a use of a stem cell selected according to a method of any of the previous aspects of the invention in a method of treating an individual. Applications of stem cells include therapeutic applications which are reviewed for example in Nature Insight Review, Vol 414, November 2001. In particular stem cells may be targets for gene therapy and may be genetically modified prior to their use in therapeutic applications as described, for example, in Rideout et al. Cell, 109(1):17-27, 2002; Wu et al. Gene Ther 9(4), 245-255, Feb 2002; Lebkowski et al. Cancer J. 7 Suppl 2; S83-93; Nov-Dec 2001.

In another aspect, the methods of the invention may be applicable to confirming the absence of 5T4 negative i.e. undifferentiated cells from a population prior to introducing said cells into an individual.

In a further aspect of the invention there is provided an isolated antibody recognising the membrane proximal extracellular domain of murine 5T4. Suitably, said antibody is an isolated rat monoclonal anti-5T4 antibody, 9A7.

Other aspects of the present invention are presented in the accompanying claims and in the following description and discussion. These aspects are presented under separate section headings. However, it is to be understood that the teachings under each section heading are not necessarily limited to that particular section heading.

Detailed Description of the Invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, nucleic acid chemistry, hybridisation techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods. See, generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.; as well as Guthrie et al., Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, Vol. 194, Academic Press, Inc., (1991), PCR Protocols: A Guide to Methods and Applications (Innis, et al. 1990. Academic Press, San Diego, Calif.), McPherson et al., PCR Volume 1, Oxford University Press, (1991), Culture of Animal Cells: A Manual of Basic Technique, 2nd Ed. (R. I. Freshney. 1987. Liss, Inc. New York, N.Y.), and Gene Transfer and Expression Protocols, pp. 109-128, ed. E. J. Murray, The Humana Press Inc., Clifton, N.J.). These documents are incorporated herein by reference.

5T4 antigen is the polypeptide known as 5T4 and characterised, for example, in WO89/07947. "5T4" may be human 5T4 as characterised by Myers *et al ibid.*, the sequence of which appears in GenBank at accession no. Z29083. A sequence for mouse or murine 5T4 (m5T4) appears in GenBank at Accession no. AJ012160. The organisation of the mouse and human 5T4 genes is described, for example, by King et al. Biochim Biophys Acta 1999; 1445 (3); 257-70. Canine and feline 5T4 sequences are described, for example, in PCT/GB01/05004 (WO 02/38612).

Sequence analysis of the human 5T4 cDNA identified the antigen as a member of the leucine rich repeat (LRR) family of proteins (Myers, K. A. et al. (1994)). The protein contains a short cytoplasmic tail of 44 amino acids and an extracellular domain consisting of two leucine rich repeat (LRR) regions with associated cysteine containing flanking regions and separated by a hydrophilic domain. All of the seven consensus NxS/T N-glycosylation sites in the extracellular domain are glycosylated with a combination of complex glycans, including two high mannose chains and five sialylated, bi- to tetra-antennary complex chains with minor quantities of core fucosylation (Shaw, D. M. et al. (2002)).

LRR proteins are a diverse family of approximately 60 members, which have in common a repeating structure of aXXaXaXXN/C/T, where a is an aliphatic residue such as leucine and X is any amino acid (Kobe et al. (1994)). The tertiary structure of porcine ribonuclease inhibitor, which is comprised entirely of LRRs, has been solved by X-ray crystallography (Kobe et al. (1994)). Ribonuclease inhibitor folds into a horseshoe-like structure of repeating units of α-helix and β-pleated sheets, this resolved structure has formed the basis of structural models for other family members (Kajava et al. (1995); Janosi et al. (1999)). However, the precise structure may vary due to differences in the lengths of the LRRs and the presence of other functional domains. Despite no common function having been ascribed, many are involved in protein-protein interactions and overall it is likely that the LRR domains provide a scaffold for a variety of functions (Kobe et al. (1994), Kobe et al. (1995)).

5T4 antigen is expressed on microvillus projections of cells and when the human 5T4 cDNA is constitutively overexpressed in certain fibroblasts or epithelial cells, there are alterations in motility and morphology which are consistent with a role in both tumour and trophoblast invasion (Carsberg et al. (1995); Carsberg et al. (1996)).

Sequence comparisons between the human and mouse 5T4 cDNAs (King et al. (1999)) indicates the highly conserved structure of 5T4 molecules between species. These molecules share 81% amino acid identity, with the cytoplasmic and transmembrane domains being completely conserved. Of the seven N-linked glycosylation sites in the human molecule, six are conserved in the mouse. The most N-terminal site (N81) is absent, but an additional site (N334) in the C-terminal flanking region is present predicting a similar level of glycosylation to the human molecules. The murine protein contains an additional six amino acids adjacent to the glycosylation site in the hydrophilic domain, which is a direct repeat of the preceding six amino acids. The expression of 5T4 in trophoblasts suggests it is present at a stage of development common to all mammals. This makes it likely that 5T4 is highly conserved throughout mammals.

As used herein, "undifferentiated cells" with particular reference to stem cells means cells which retain their characteristic pluripotency or multipotency i.e. their ability to give rise to all cell types or more than one differentiated cell type. The terms "differentiated" or "differentiation status" when referring to a cell means cells that have begun to or have partially or completely developed into cells with a defined phenotype. The characteristic phenotypes of particular

differentiated cell types are dependent on the particular cell type and are recognised to those skilled in the art.

As used herein, the term "polypeptide" refers to a polymer in which the monomers are amino acids and are joined together through peptide or disulphide bonds. "Polypeptide" refers to a full-length naturally-occurring amino acid chain or a fragment thereof, such as a selected region of the polypeptide that is of interest in a binding interaction, or a synthetic amino acid chain, or a combination thereof. "Fragment thereof" thus refers to an amino acid sequence that is a portion of a full-length polypeptide, between about 8 and about 500 amino acids in length, preferably about 8 to about 300, more preferably about 8 to about 200 amino acids, and even more preferably about 10 to about 50 or 100 amino acids in length. Additionally, amino acids other than naturally-occurring amino acids, for example ß-alanine, phenyl glycine and homoarginine, may be included. Commonly-encountered amino acids which are not gene-encoded may also be used in the present invention.

The expression "5T4 antigen" encompasses fragments thereof, and preferably those fragments having distinct epitopes, and variants thereof comprising amino acid insertions, deletions or substitutions which retain the antigenicity of 5T4. Suitably, the term 5T4 antigen, includes peptides and other fragments of 5T4 which retain at least one common antigenic determinant of 5T4.

"Common antigenic determinant" means that the derivative in question has at least one antigenic function of 5T4. Antigenic functions includes possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against a naturally occurring or denatured 5T4 polypeptide or fragment thereof, or the ability to bind HLA molecules and induce a 5T4-specific immune response.

Thus 5T4 antigen as referred to herein includes amino acid mutants, glycosylation variants and other covalent derivatives of 5T4 which retain the physiological and/or physical properties of 5T4. Exemplary derivatives include molecules wherein the protein of the invention is covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a radioisotope. Further included are naturally occurring variants of 5T4 found with a particular species, preferably a mammal. Such a

variant may be encoded by a related gene of the same gene family, by an allelic variant of a particular gene, or represent an alternative splicing variant of the 5T4 gene.

Derivatives which retain common antigenic determinants can be fragments of 5T4. Fragments of 5T4 comprise individual domains thereof, as well as smaller polypeptides derived from the domains. Preferably, smaller polypeptides derived from 5T4 according to the invention define a single epitope which is characteristic of 5T4. Fragments may in theory be almost any size, as long as they retain one characteristic of 5T4. Preferably, fragments will be between 5 and 400 amino acids in length. Longer fragments are regarded as truncations of the full-length 5T4 and generally encompassed by the term "5T4". Advantageously, fragments are relatively small peptides of the order of 5 to 25 amino acids in length. Preferred are peptides about 9 amino acids in length.

Derivatives of 5T4 also comprise mutants thereof, which may contain amino acid deletions, additions or substitutions, subject to the requirement to maintain at least one feature characteristic of 5T4. Thus, conservative amino acid substitutions may be made substantially without altering the nature of 5T4, as may truncations from the 5' or 3' ends. Deletions and substitutions may moreover be made to the fragments of 5T4 comprised by the invention. 5T4 mutants may be produced from a DNA encoding 5T4 which has been subjected to *in vitro* mutagenesis resulting e.g. in an addition, exchange and/or deletion of one or more amino acids. For example, substitutional, deletional or insertional variants of 5T4 can be prepared by recombinant methods and screened for immuno-crossreactivity with the native forms of 5T4.

The fragments, mutants and other derivatives of 5T4 preferably retain substantial homology with 5T4. As used herein, "homology" means that the two entities share sufficient characteristics for the skilled person to determine that they are similar in origin and function.

"Substantial homology", where homology indicates sequence identity, means more than 40% sequence identity, preferably more than 45% sequence identity and most preferably a sequence identity of 50% or more, as judged by direct sequence alignment and comparison.

Sequence homology (or identity) may moreover be determined using any suitable homology algorithm, using for example default parameters. Advantageously, the BLAST algorithm is employed, with parameters set to default values. The BLAST algorithm is described in detail

at http://www.ncbi.nih.gov/BLAST/blast_help.html, which is incorporated herein by reference.

As used herein, the term "antibody" refers to a polypeptide, at least a portion of which is encoded by at least one immunoglobulin gene, or fragment thereof, and that can bind specifically to a desired target molecule. The term includes naturally-occurring forms, as well as fragments and derivatives.

"Specific binding" refers to the ability of two molecular species concurrently present in a heterogeneous (inhomogeneous) sample to bind to one another in preference to binding to other molecular species in the sample. Typically, a specific binding interaction will discriminate over adventitious binding interactions in the reaction by at least two-fold, more typically by at least 10-fold, often at least 100-fold; when used to detect analyte, specific binding is sufficiently discriminatory when determinative of the presence of the analyte in a heterogeneous (inhomogeneous) sample.

As used herein, a "vector" may be any agent capable of delivering or maintaining nucleic acid in a host cell, and includes viral vectors, plasmids, naked nucleic acids, nucleic acids complexed with polypeptide or other molecules and nucleic acids immobilised onto solid phase particles.

A "nucleic acid", as referred to herein, may be DNA or RNA, naturally-occurring or synthetic, or any combination thereof. Nucleic acids encoding 5T4 antigen may be constructed in such a way that it may be translated by the machinery of the cells of a host organism. Thus, natural nucleic acids may be modified, for example to increase the stability thereof. DNA and/or RNA, but especially RNA, may be modified in order to improve nuclease resistance. For example, known modifications for ribonucleotides include 2'-O-methyl, 2'-fluoro, 2'-NH_2, and 2'-O-allyl. Modified nucleic acids may comprise chemical modifications which have been made in order to increase the *in vivo* stability of the nucleic acid, enhance or mediate the delivery thereof, or reduce the clearance rate from the body. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions of a given RNA sequence. See, for example, WO 92/03568; U.S. 5,118,672; Hobbs *et al.*, (1973) Biochemistry 12:5138; Guschlbauer *et al.*, (1977) Nucleic

Acids Res. 4:1933; Schibaharu *et al.*, (1987) Nucleic Acids Res. 15:4403; Pieken *et al.*, (1991) Science 253:314, each of which is specifically incorporated herein by reference.

Methods of detecting 5T4 expression

The term "expression" refers to the transcription of a gene's DNA template to produce the corresponding mRNA and translation of this mRNA to produce the corresponding gene product (*i.e.*, a peptide, polypeptide, or protein). 5T4 antigen is "expressed" in accordance with the present invention by being produced in the cells as a result of translation, and optionally transcription, of the nucleic acid encoding 5T4. Thus, 5T4 is produced *in situ* in the cell. Since 5T4 is a transmembrane protein, the extracellular portion thereof is displayed on the surface of the cell in which it is produced.

a) at the RNA level

Expression levels can be assessed by measuring gene transcription. This is preferably carried out by measuring the rate and/or amount of specific mRNA production in the cell. RNA may be extracted from cells using RNA extraction techniques including, for example, using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), or RNeasy RNA preparation kits (Qiagen). Typical assay formats utilising ribonucleic acid hybridisation include nuclear run-on assays, RT-PCR and RNase protection assays (Melton et al., Nuc. Acids Res. 12:7035). Methods for detection which can be employed include radioactive labels, enzyme labels, chemiluminescent labels, fluorescent labels and other suitable labels.

Typically, RT-PCR is used to amplify RNA targets. In this process, the reverse transcriptase enzyme is used to convert RNA to complementary DNA (cDNA) which can then be amplified to facilitate detection.

Many DNA amplification methods are known, most of which rely on an enzymatic chain reaction (such as a polymerase chain reaction, a ligase chain reaction, or a self-sustained sequence replication) or from the replication of all or part of the vector into which it has been cloned.

Many target and signal amplification methods have been described in the literature, for example, general reviews of these methods in Landegren, U., et al., Science 242:229-237 (1988) and Lewis, R., Genetic Engineering News 10:1, 54-55 (1990).

PCR is a nucleic acid amplification method described inter alia in U.S. Pat. Nos. 4,683,195 and 4,683,202. PCR can be used to amplify any known nucleic acid in a diagnostic context (Mok et al., (1994), Gynaecologic Oncology, 52: 247-252).

A number of alternative amplification technologies including rolling circle amplification (Lizardi et al., (1998) Nat Genet 19:225) are known to those skilled in the art.

A primer may be used to allow specific amplification of 5T4 mRNA. A probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most preferably at least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases of the mRNA of interest.

Primers suitable for use in various amplification techniques can be prepared according to methods known in the art.

Once the nucleic acid has been amplified, a number of techniques are available for the quantification of DNA and thus quantification of the RNA transcripts present. Methods for detection which can be employed include radioactive labels, enzyme labels, chemiluminescent labels, fluorescent labels and other suitable labels.

Probes may be used to detect the presence of their corresponding sequences through hybridisation reactions e.g. in blotting techniques such as northern or southern blotting. The presence of 5T4 nucleic acid sequences may be detected by hybridisation with specific 5T4 probes under stringent conditions.

The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of 5T4.

Either the full-length cDNA for 5T4 or fragments thereof can be used as probes. Preferably, nucleic acid probes are labeled with suitable label means for ready detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labeling a DNA fragment is by incorporating α32P dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labeled with γ32P-labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g. enzyme labelling, fluorescent labelling with suitable fluorophores and biotinylation.

Stringency of hybridisation refers to conditions under which polynucleic acid hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (Tm) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M Na+ at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na+ pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

Moderate stringency refers to conditions equivalent to hybridisation in the above described solution but at about 60-62°C. In that case the final wash is performed at the hybridisation temperature in 1x SSC, 0.1 % SDS.

Low stringency refers to conditions equivalent to hybridisation in the above described solution at about 50-52°C. In that case, the final wash is performed at the hybridisation temperature in 2x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, *et al.*, eds. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York or Ausubel, *et al.*, eds. (1990) Current Protocols in Molecular Biology, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

In the context of the present invention, detection of 5T4 expression gives an indication of differentiation status of mammalian ES cells where an increase in 5T4 transcription is an indication of induction of differentiation whereas the absence, or expression at low or negligible levels is an indication of undifferentiated status.

b) at the protein level

Gene expression may also be detected at the protein level by measuring amounts of 5T4 antigen polypeptide. A variety of protocols for detecting and measuring the expression of the amino acid sequences are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 15 8:121 1). A suitable FACS-based method is described in the Examples section herein.

Detection of protein expression may be achieved by using molecules which bind to the 5T4 antigen polypeptide. Suitable molecules/agents which bind either directly or indirectly to 5T4 in order to detect the presence of the protein include naturally occurring molecules such as peptides and proteins, for example antibodies, or they may be synthetic molecules.

Other naturally occurring molecules which bind 5T4 include specific 5T4 ligands. For example, a number of intracellular partners for 5T4 have been identified and are described in Awan et al. (Biochem Biophys Res Comm (2002); 290 (3); 1030-1036).

Anti-5T4 antibodies are antibodies that specifically bind to 5T4 antigen. They may be polyclonal or monoclonal. If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing a 5T4 epitope such as 5T4-Fc. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to a 5T4 epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. Such antibodies may also be made using polypeptides or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

An immune response may also be elicited by immunisation with a vector comprising a 5T4-expressing nucleic acid.

The vector employed for immunisation may be any vector, viral or non-viral. The 5T4 antigen used, whether full length 5T4 or peptides thereof, may be modified and may be homologous (i.e. derived from the same species as the subject stem cells) or heterologous in origin.

Monoclonal antibodies directed against 5T4 epitopes can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against 5T4 epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv).

Standard laboratory techniques involving antibodies can be used to detect levels of 5T4 in stem cells. One such technique is immunoblotting, an example of a suitable protocol for which is detailed below:

Aliquots of total protein extracts from stem cells (40μg), are run on SDS-PAGE and electroblotted overnight at 4°C onto nitrocellulose membrane. Immunodetection involves antibodies specific for 5T4, appropriate secondary antibodies (goat, anti-rabbit or goat-anti-mouse: Bio-Rad, CA, USA) conjugated to horseradish peroxidase, and the enhanced ECL chemiluminescence detection system (Amersham, UK).

Methods for selecting cells by 5T4 expression

A variety of selection procedures may be applied for the isolation of cells expressing 5T4 (positive selection) or undifferentiated cells lacking 5T4 expression (negative expression). These include Fluorescence Activated Cell Sorting (FACS), cell separation using magnetic particles, panning, antigen chromatography methods and other cell separation techniques such as use of polystyrene beads.

Separating cells using magnetic capture may be accomplished by conjugating a molecule which binds to 5T4 antigen to magnetic particles or beads. For example, the 5T4 binding agent may be conjugated to superparamagnetic iron-dextran particles or beads as supplied by Miltenyi Biotec GmbH.. These conjugated particles or beads are then mixed with a cell population which may express 5T4. If a particular cell expresses 5T4, it will become complexed with the magnetic beads by virtue of this interaction. A magnetic field is then applied to the suspension which immobilises the magnetic particles, and retains any cells which are associated with them via the covalently linked antigen. Unbound cells which do not become linked to the beads can be washed away or collected separately, leaving a population of cells which is isolated by virtue of the expression of 5T4. Reagents and kits are available from various sources for performing such isolations, and include Dynal Beads (Dynal AS; http://www.dynal.no), MACS-Magnetic Cell Sorting (Miltenyi Biotec GmbH; http://www.miltenyibiotec.com), CliniMACS (AmCell; http://www.amcell.com) as well as Biomag, Amerlex-M beads and others.

Fluorescence Activated Cell Sorting (FACS) can be used to isolate cells on the basis of their differing surface molecules, for example surface displayed 5T4. Cells in the sample or population to be sorted are stained with specific fluorescent reagents which bind to 5T4. These reagents would be the 5T4 binding agent linked (either directly or indirectly) to fluorescent markers such as fluorescein, Texas Red, malachite green, green fluorescent protein (GFP), or any other fluorophore known to those skilled in the art. The cell population is then introduced into the vibrating flow chamber of the FACS machine. The cell stream passing out of the chamber is encased in a sheath of buffer fluid such as PBS (Phosphate Buffered Saline). The stream is illuminated by laser light and each cell is measured for fluorescence, indicating binding of the fluorescent labelled antigen. The vibration in the cell stream causes it to break up into droplets, which carry a small electrical charge. These droplets can be steered by electric deflection plates under computer control to collect different cell populations according to their affinity for the fluorescent labelled binding agent. In this manner, cell populations which express 5T4 can be easily separated from those cells which do not express 5T4. FACS machines and reagents for use in FACS are widely available from sources world-wide such as Becton-Dickinson, or from service providers such as Arizona Research Laboratories (http://www.arl.arizona.edu/facs/).

Another method which can be used to separate populations of cells according to cell surface expression of 5T4 is affinity chromatography. In this method, a suitable resin (for example CL-600 Sepharose, Pharmacia Inc.) is covalently linked to the appropriate 5T4 binding agent. This resin is packed into a column, and the mixed population of cells is passed over the column. After a suitable period of incubation (for example 20 minutes), unbound cells are washed away using (for example) PBS buffer. This leaves only that subset of cells expressing 5T4 and these cells are then eluted from the column using (for example) an excess of the 5T4, or by enzymatically or chemically cleaving the bound reagent from the resin thereby releasing that population of cells which exhibited 5T4 expression.

Expression from the 5T4 promoter

The term "promoter" or "promoter region" refers to a nucleic acid sequence, usually found upstream (5') to a coding sequence, that is capable of directing transcription of a nucleic acid sequence into mRNA. The promoter or promoter region typically provide a recognition site for RNA polymerase and the other factors necessary for proper initiation of transcription. As

contemplated herein, a promoter or promoter region includes variations of promoters derived by inserting or deleting regulatory regions, subjecting the promoter to random or site-directed mutagenesis, *etc.* The activity or strength of a promoter may be measured in terms of the amounts of RNA it produces, or the amount of protein accumulation in a cell or tissue, relative to a promoter whose transcriptional activity has been previously assessed.

A "nucleic acid encoding the promoter sequence of 5T4" means a nucleic acid sequence which is capable of directing endogenous transcription of 5T4 gene expression. The term moreover includes those polynucleotides capable of hybridising, under stringent hybridisation conditions, to the naturally occurring nucleic acids identified above, or the complement thereof.

The phrase "operably linked" refers to the functional spatial arrangement of two or more nucleic acid regions or nucleic acid sequences. For example, a promoter region may be positioned relative to a nucleic acid sequence such that transcription of a nucleic acid sequence is directed by the promoter region. Thus, a promoter region is "operably linked" to the nucleic acid sequence.

A "reporter gene" is a gene which is incorporated into an expression vector and placed under the same controls as a gene of interest to express an easily measurable phenotype.

Methods for detecting transcription from a promoter sequence

Transcription from the 5T4 promoter sequence can be detected using a nucleic acid construct comprising the 5T4 promoter sequence operably linked to a reporter gene. A "reporter gene" is a gene which is incorporated into an expression vector and placed under the same controls as a gene of interest to express an easily measurable phenotype. A number of suitable reporter genes are known whose expression may be detectable by histochemical staining, liquid scintillation, spectrophotometry or luminometry. Many reporters have been adapted for a broad range of assays, including colorimetric, fluorescent, bioluminescent, chemiluminescent, ELISA, and/or in situ staining. Suitable reporter systems are based on the expression of enzymes such as chloramphenicol acetyltransferase (CAT), b-galatosidase (b-gal), b-glucuronidase, alkaline phosphatase and luciferase. More recently, a number of reporter systems have been developed which are based on using Green fluorescent proteins (GFP) or various derivatives or mutant forms including EGFP. Reporter genes and detection systems

are reviewed by Sussman in The Scientist 15[15]:25, Jul. 23, 2001 which is incorporated by reference.

Vectors for gene delivery or expression .

To generate cells expressing an exogenous gene or 5T4-expressing cells, polypeptides such as 5T4 polypeptides can be delivered by viral or non-viral techniques. Delivery of 5T4 antigen for immunisation purposes can also be through viral or non-viral techniques.

Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a 5T4 gene to a target mammalian cell. The post-translational modification in relation to phosphorylation or glycosylation may be varied by expression of 5T4 in different target cells.

Typical transfection methods include electroporation, nucleic acid biolistics, lipid-mediated transfection, compacted nucleic acid-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), multivalent cations such as spermine, cationic lipids or polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (Wolff and Trubetskoy 1998 Nature Biotechnology 16: 421) and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, retroviral vectors, lentiviral vectors or baculoviral vectors, venezuelan equine encephalitis virus (VEE), poxviruses such as: canarypox virus (Taylor *et al 1995* Vaccine 13:539-549), entomopox virus (Li Y *et al* 1998 XIIth International Poxvirus Symposium p144. Abstract), penguine pox (Standard *et al.* J Gen Virol. 1998 79:1637-46) alphavirus, and alphavirus based DNA vectors.

A detailed list of retroviruses may be found in Coffin *et al* ("Retroviruses" 1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763).

Lentiviruses can be divided into primate and non-primate groups. Examples of primate lentiviruses include but are not limited to: the human immunodeficiency virus (HIV), the

causative agent of human auto-immunodeficiency syndrome (AIDS), and the simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

A distinction between the lentivirus family and other types of retroviruses is that lentiviruses have the capability to infect both dividing and non-dividing cells (Lewis *et al* 1992 EMBO. J 11: 3053-3058; Lewis and Emerman 1994 J. Virol. 68: 510-516). In contrast, other retroviruses - such as MLV - are unable to infect non-dividing cells such as those that make up, for example, muscle, brain, lung and liver tissue.

The vector encoding 5T4 may be configured as a split-intron vector. A split intron vector is described in PCT patent applications WO 99/15683 and WO 99/15684.

If the features of adenoviruses are combined with the genetic stability of retroviruses/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that could stably infect neighbouring cells. Such retroviral producer cells engineered to express 5T4 antigen can be implanted in organisms such as animals or humans for use in the treatment of angiogenesis and/or cancer.

Pox viruses are engineered for recombinant gene expression and for the use as recombinant live vaccines. This entails the use of recombinant techniques to introduce nucleic acids encoding foreign antigens into the genome of the pox virus. If the nucleic acid is integrated at a site in the viral DNA which is non-essential for the life cycle of the virus, it is possible for the newly produced recombinant pox virus to be infectious, that is to say to infect foreign cells and thus to express the integrated DNA sequence. The recombinant pox virus prepared in this way can be used as live vaccines for the prophylaxis and/or treatment of pathologic and infectious disease. Such live vaccines can also be used to raise antibodies against 5T4. Suitable vectors derived from Vaccinia Western Reserve are described in the Examples section herein.

Expression of 5T4 in recombinant pox viruses, such as vaccinia viruses, requires the ligation of vaccinia promoters to the nucleic acid encoding 5T4. Plasmid vectors (also called insertion

vectors), have been constructed to insert nucleic acids into vaccinia virus through homologous recombination between the viral sequences flanking the nucleic acid in a donor plasmid and homologous sequence present in the parental virus (Mackett *et al* 1982 PNAS 79: 7415-7419). One type of insertion vector is composed of: (a) a vaccinia virus promoter including the transcriptional initiation site; (b) several unique restriction endonuclease cloning sites located downstream from the transcriptional start site for insertion of nucleic acid; (c) nonessential vaccinia virus sequences (such as the Thymidine Kinase (*TK*) gene) flanking the promoter and cloning sites which direct insertion of the nucleic acid into the homologous nonessential region of the virus genome; and (d) a bacterial origin of replication and antibiotic resistance marker for replication and selection in *E. Coli*. Examples of such vectors are described by Mackett (Mackett *et al* 1984, J. Virol. 49: 857-864).

The isolated plasmid containing the nucleic acid to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the parental virus, e.g., poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively results in a recombinant poxvirus modified by the presence of the promoter-gene construct in its genome, at a site which does not affect virus viability.

As noted above, the nucleic acid is inserted into a region (insertion region) in the virus which does not affect virus viability of the resultant recombinant virus. Such regions can be readily identified in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase (TK) gene. For example, the TK gene has been found in all pox virus genomes examined [leporipoxvirus: Upton, *et al* J. Virology 60:920 (1986) (shope fibroma virus); capripoxvirus: Gershon, *et al* J. Gen. Virol. 70:525 (1989) (Kenya sheep-1); orthopoxvirus: Weir, *et al* J. Virol 46:530 (1983) (vaccinia); Esposito, *et al* Virology 135:561 (1984) (monkeypox and variola virus); Hruby, *et al* *PNAS*, 80:3411 (1983) (vaccinia); Kilpatrick, *et al* Virology 143:399 (1985) (Yaba monkey tumour virus); avipoxvirus: Binns, *et al* J. Gen. Virol 69:1275 (1988) (fowlpox); Boyle, *et al* Virology 156:355 (1987) (fowlpox); Schnitzlein, *et al* J. Virological Method, 20:341 (1988) (fowlpox, quailpox); entomopox (Lytvyn, *et al* J. Gen. Virol 73:3235-3240 (1992)].

In vaccinia, in addition to the TK region, other insertion regions include, for example, HindIII M.

In fowlpox, in addition to the TK region, other insertion regions include, for example, BamHI J [Jenkins, *et al* AIDS Research and Human Retroviruses 7:991-998 (1991)] the *Eco*RI-*Hind*III fragment, *Bam*HI fragment, *Eco*RV-*Hind*III fragment, *Bam*HI fragment and the HindIII fragment set forth in EPO Application No. 0 308 220 A1. [Calvert, *et al* J. of Virol 67:3069-3076 (1993); Taylor, *et al* Vaccine 6:497-503 (1988); Spehner, *et al* (1990) and Boursnell, *et al* J. of Gen. Virol 71:621-628 (1990)].

In swinepox preferred insertion sites include the thymidine kinase gene region.

A promoter can readily be selected depending on the host and the target cell type. For example in poxviruses, pox viral promoters should be used, such as the vaccinia 7.5K, or 40K or fowlpox C1. Artificial constructs containing appropriate pox sequences can also be used. Enhancer elements can also be used in combination to increase the level of expression. Furthermore, the use of inducible promoters, which are also well known in the art, are preferred in some embodiments.

Foreign gene expression can be detected by enzymatic or immunological assays (for example, immuno-precipitation, radioimmunoassay, or immunoblotting). Naturally occurring membrane glycoproteins produced from recombinant vaccinia infected cells are glycosylated and may be transported to the cell surface. High expressing levels can be obtained by using strong promoters.

Stem cells

Stem cells are undifferentiated, primitive cells with the ability both to multiply and differentiate into specific kinds of cells. Mammalian stem cells can be pluripotent cell lines derived from mammalian embryos, such as ES, EG or EC cells, or can be multipotent and derived from adults. Adult-derived stem cells include neural stem cells, mesenchymal stem cells, hematopoeitic stem cells and epithelial stem cells. Stem cell cultures may be genetically modified after isolation and prior to their differentiation.

Mammalian stem cells may be derived from any mammalian species and thus may be murine, human or other primate (e.g. chimpanzee, cynomolgus monkey, baboon, other Old World monkey), porcine, canine, equine, feline etc.

Embryonic stem (ES) cells are stem cells derived from the pluripotent inner cell mass (ICM) cells of the pre-implantation, blastocyst-stage embryo. Outgrowth cultures of blastocysts give rise to different types of colonies of cells, some of which have an undifferentiated phenotype. If these undifferentiated cells are sub-cultured onto feeder layers they can be expanded to form established ES cell lines that seem immortal. These pluripotent stem cells can differentiate in vitro into a wide variety of cell types representative the three primary germ layers in the embryo. Methods for deriving ES cells are known for example from Evans et al. 1981; Nature; 29; 154-156.

Embryonic germ (EG) cell lines are derived from primordial germ cells. Methods for the isolation and culture of these cells are described, for example, by McLaren et al. Reprod. Fertil. Dev 2001; 13 (7-8):661-4. Other types of stem cells include embryonal carcinoma cells (EC) (as reviewed, for example, in Donovan and Gearhar, Nature 2001; Insight review article p 92 – 97).

Other types of stem cells include cells having haploid genomes as described, for example, in WO 01/32015.

Methods for isolating human pluripotent stem cells are described, for example, by Trounson, A. O. Reprod. Fertil. Dev 2001; 13 (7-8): 523-32. Isolation requires feeder cells (and 20% fetal calf serum) or conditioned medium from feeder cells. Further methods for producing pluripotent cells are known from WO 01/30978 where the derivation of pluripotent cells from oocytes containing DNA of all male or female origin is described. In addition, stem cell-like lines may be produced by cross species nuclear transplantation as described, for example, in WO 01/19777, by cytoplasmic transfer to de-differentiate recipient cells as described, for example, in WO 01/00650 or by "reprogramming" cells for enhanced differentiation capacity using pluripotent stem cells (see WO 02/14469).

Stem Cell Culture

Cell culture conditions may be modified to favour maintenance of the cells in an undifferentiated state. If conditions are not carefully selected, stem cells may follow their natural capacity to differentiate into other cells. ES cells, for example, may differentiate into cells resembling those of extraembryonic lineages. Few of the factors that regulate self-renewal of pluripotent stem cells are currently known. Typically, pluripotent stem cell lines are isolated and maintained on mitotically inactive feeder layers of fibroblasts.

Typically, culture systems for ES cells comprise the use of media such as Dulbecco's modified Eagle's medium (DMEM) as a basal media with the addition of amino acids and beta mercaptoethanol, serum supplementation (normally Fetal Calf Serum (FCS)), and a embryonic mesenchymal feeder cell support layer. Basal media and serum supplements can be obtained from a number of commercial sources. However, any media or serum is subject to variability and even small variations can effect the ES cell culture conditions.

Cells maintained in their undifferentiated state may be subjected to control differentiating conditions to generate cells of the desired somatic lineage. Cultured stem cells can be induced to differentiate by separation of stem cells from feeder cells or by growth of stem cell colonies in suspension culture to form embryoid bodies which upon dissociation can be plated to yield differentiating cells. Conditions for obtaining differentiated cultures of somatic cells from ES cells are described, for example, in PCT/AU99/00990. Leukaemia inhibitory factor (LIF) has been identified as one of the factors that can maintain pluripotent stem cells; LIF can replace the requirement for feeder cells for murine ES cells (see Nichols et al.; (1990) Development 110; 1341-1348). Differentiation by removal of LIF is described herein.

Modulating 5T4 expression or activity

The "functional activity" of a protein in the context of the present invention describes the function the protein performs in its native environment. Altering or modulating the functional activity of a protein includes within its scope increasing, decreasing or otherwise altering the native activity of the protein itself. In addition, it also includes within its scope increasing or decreasing the level of expression and/or altering the intracellular distribution of the nucleic acid encoding the protein, and/or altering the intracellular distribution of the protein itself.

The functional activity of 5T4 may be modified by suitable molecules/agents which bind either directly or indirectly to 5T4, or to the nucleic acid encoding it. Agents may be naturally occurring molecules such as peptides and proteins, for example antibodies, or they may be synthetic molecules. Methods of modulating the level of expression of 5T4 include, for example, using antisense techniques. Antisense constructs are described in detail in US 6,100,090 (Monia et al), and Neckers et al., 1992, *Crit Rev Oncog* 3(1-2):175-231, the teachings of which documents are specifically incorporated by reference. Other methods of modulating gene expression are known to those skilled in the art and include dominant negative approaches as well as introducing peptides or small molecules which inhibit gene expression or functional activity.

Uses of stem cells

A number of applications for stem cells are known. For example, ES cells may be used as an in vitro model for differentiation, especially for the study of genes which are involved in the regulation of early development. ES cells also have potential utility for germline manipulation of livestock animals by using ES cells with or without a desired genetic mutation.

The therapeutic uses of mammalian stem cells are reviewed, for example, in Lovell-Badge, Nature Insight Review, November 2001, 88-91. Some types of human stem cells, such as bone marrow and skin have been used in therapies for leukemia or skin replacement while others are being used in trials including fetal midbrain cells for Parkinson's disease, and pancreatic duct cells for diabetes.

Mammalian ES cells are the easiest types of stem cells to grow in culture. A number of uses for mouse ES cells have been demonstrated in animal models (as reviewed in Donovan and Gearhart, 2001) and include generation of cardiomyocytes to form functioning intracardiac grafts, generation of myelin from glial precursors and the introduction of a genetically modified insulin-producing ES cell line to normalise glycaemia. Initial results from studies using human pluripotent stem cells in animal models suggest that neuronal cells may be useful in treatment of stroke patient whereas there are number of potential applications for mesenchymal-derived stem cells including cardiac muscle repair, bone regeneration and joint repair.

The invention is further described, for the purposes of illustration only, in the following examples in which reference is made to the following Figures and Tables:

Table 1 shows the results of FACS analysis of 9A7 activity against a panel of murine cell lines. 10^5 cells of each line were stained with 9A7 and analysed by FACS. Results are representative of three individual cultures and staining experiments.

Table 2. Expression of m5T4 antigen mediates a reduced mean cell volume. FACS was used to assess the forward scatter profile of mid-log phase cultures of the cell lines listed. The geometric mean of the forward scatter was taken as a measure of average cell volume. These results are representative of three separate experiments.

Figure 1. The Rabbit anti-m5T4 polyclonal antisera is specific for m5T4 by FACS. Panel A shows the effect of a decreasing concentration m5T4-Fc upon the binding of a constant concentration of Rabαm5T4 to B16 F10-m5T4 cells. Cells were analysed by FACS and results expressed as a percentage of the maximal geometric mean. Panels B-D; Grey profiles show A9-m5T4 transfectants stained with Rabαm5T4 (1:300 B-D). White profiles show A9-m5T4 (B -rabbit pre-immune serum 1:300), A9H12 neomycin control (C -Rabαm5T4 1:300) and A9-h5T4 (D- Rabαm5T4 1:300).

Figure 2. Specificity of the 9A7 antibody for m5T4 cDNA transfected cells by FACS. Grey profiles show A9-m5T4 (9A7, A-C). White profiles show A9m5T4 (rat IgG, A), and A9H12 neomycin (9A7, B), A9-h5T4 transfectants (9A7, C). Panel D shows the effect of a decreasing concentration of human or mouse 5T4-Fc upon the ability of a constant concentration of 9A7 to stain A9m5T4 cells. Cells were analysed by FACS and results expressed as a percentage of the maximal geometric mean.

Figure 3. 9A7 is specific for m5T4 by ELISA. The capacity of various antigens to inhibit the binding of 9A7 to m5T4-Fc was investigated. Antigen was titrated in a constant concentration of 9A7 (1μg/ml) and immediately applied to m5T4-Fc coated plates (1μg/ml).

Figure 4. The 9A7 epitope maps to the membrane proximal region of m5T4. A9 cell lines expressing human-mouse 5T4 (A and Ci) or mousehuman 5T4 chimeric cDNA constructs (B and Cii), in a stable manner, were labelled with 9A7 (grey profiles) or MAb 5T4 (white profiles). Panel C shows a diagrammatic representation of the 5T4 chimeric molecules. Mouse sequences are shown in grey and human sequences in black. From the amino terminus the domains are labelled; N (amino terminal flanking region), LRR1 (leucine rich region repeat 1), HP (hydrophilic region), LRR2 (leucine rich region repeat 2), C (C terminal flanking region), TM (trans-membrane region) and CYT (cytoplasmic domain).

Figure 5. Biochemical analysis of 9A7 specificity by Western blot. Panel A: Lanes were loaded with 50ng of human (h) or mouse (m) 5T4-Fc fusion protein under non-reducing (i) or reducing conditions (ii) and probed with a rat anti-mouse 5T4 polyclonal antisera (1:200) or 9A7 (5μg/ml). B: non-reduced Western blot of cell lysates (i) and a 9A7 imunoprecipitation (ii) from A9 cells; wild type (wt), neomycin control (neo), human (h) or mouse (m) 5T4. Cell lysates were loaded at $4x10^5$ cell equivalents per lane (i), and 10^6 cell equivalents were immunoprecipitated with 5μg of 9A7 with the entire reaction loaded (ii). Both panels (Bi-ii) were probed with rabbitαm5T4 (1:3000).

Figure 6. Distribution of m5T4 at the cell surface. A9h5T4 (A-B), A9m5T4 (C-D) and B16 F10-m5T4 (E-F) cells were pre-fixed and stained with MAb 5T4 (A-B) or 9A7 (C-F) and analysed by confocal microscopy. Panels show, the entire Z stack projection (A,C,E) or a single Z slice at midpoint of Z stack (B,D,F). Each image contains a standard 10μm bar.

Figure 7. The distribution of m5T4 after disruption of the cytoskeleton. Cells were left untreated (A) or treated with the cytoskeletal poisons Demecolcine (B) or Cytochalasin D (C) to disrupt the microtubule network or the actin fillaments respectively. 2 hours later cells were labelled with 9A7 and analysed by confocal microscopy. Each image contains a standard 10μm bar.

Figure 8. 5T4 antigen expression affects the proliferation and growth patterns of A9 cells. Panels A, B and C show typical fields of view of A9H12 Neomycin control cells (A), A9-h5T4 cells (B) and A9-m5T4 cells (C) at 200X magnification. All cultures were seeded in 10% FCS. 24hrs later the medium was changed to 1% MEM-α and cells cultured for a further two days before image capture.

Figure 9. 5T4 expression and cell adhesion. Panel A. 10^6 cells were seeded into 6 well plates in medium supplemented with 0.25, 1 and 5% FCS. 24 hours later the percentage of seeded cells attached was calculated. Panel B. Extracellular matrix proteins and adhesion. 10^3 cells were loaded into protein coated wells in serum free α-MEM containing 25μg/ml transferrin. 24 hours later wells were washed and adhesion measured by crystal violet incorporation.

Figure 10. The expression of 5T4 cDNA by A9 fibroblasts enhances their motility but does not affect their capacity to invade. The relative capacity of various A9 cell lines to pass across a Matrigel coated (A - invasion) or non-coated tissue culture inserts (B - motility) was assessed. Cells numbers were scored by measurement of incorporated crystal violet. Results are expressed as the percentage of all cells, which were present on the lower membrane.

Figure 11. Immunohistochemical analysis of murine tissues with 9A7. Transverse sections of 17.5 day mouse placenta (A-D) and longitudinal sections of adult mouse brain (E-F) were labelled with rat IgG1 (A,C,E) or 9A7 (B,D,F). Brown colouration represents antibody labelling. Images were captured at 200x magnification.

Figure 12. The 5T4 oncofoetal antigen is transcriptionally and translationally upregulated in ES cells following the removal of LIF. ES cells were differentiated for 12 days as monolayer cultures by removal of LIF from the growth medium. (a) Cell-surface 5T4 was measured using rat anti-m5T4 monoclonal antibody (open population) or control rat IgG (filled population). Viable cells were gated using forward and side scatter and the graphs show the fluorescence of this population. (b) Western blot of m5T4 using polyclonal antiserum, cells as in (a). Cells were lysed (1.2×10^7 cells/ml) and the lysate separated by unreduced SDS-PAGE. The membrane was probed using rabbit anti-m5T4 polyclonal serum followed by HRP-conjugated sheep anti-rabbit immunoglobulins and developed by enhanced chemiluminescence. Graphs show the densitometric analysis of the 5T4 bands. (c) Expression of cell-surface 5T4 in MESC ES cells differentiated for 12 days as suspended embryoid bodies, measured as in (a). (d) Effect of extended passage on cell-surface 5T4 expression in MESC ES cells, measured as in (a). (i) passage 18, (ii) passage 30. (e) Effect of cloning 129 ES cells on cell-surface 5T4 expression in 129 ES cells, assayed as in (a). (i) original population, (ii) cloned population. (f) Semi-quantitative RT-PCR (25 cycles) of 5T4 in (i) MESC (ii) D3 and (iii) OKO160 ES cell lines from day 0 (D0 - undifferentiated)

to day 12 (D12) after removal of LIF. β-tub (housekeeping gene) is included for standardisation. (g) RT-PCR (35 cycles) of 5T4 transcripts in undifferentiated (day 0) (i) MESC and (ii) OKO160 ES cell lines.

Figure 13. 5T4 expression correlates with ES cell differentiation, is expressed on all three germ layers and can be used for the optimisation of ES cell culture conditions. ES cells were differentiated for 12 days as monolayer cultures by removal of LIF from the growth medium. (a) RNA was extracted from ES cells, DNase treated and cDNA synthesised from the transcripts. RT-PCR was performed using 0.5 μg cDNA and 35 cycles on (i) MESC, (ii) D3, (iii) OKO160 and (iv) 129 ES cells. β-tub (housekeeping gene) is included for comparison purposes. mRNA is RT-PCR using β-tub primers without prior formation of cDNA to ensure the absence of genomic DNA. See Table 1 for description of markers used. (b) Determination of the Forssman antigen on differentiating (i) MESC, (ii) D3, (iii) OKO160 and (iv) 129 ES cells. Forssman antigen was measured using rat anti-Forssman antibody (open population) or control rat IgG (closed population), and detected as described in Figure 1a. (c) RT-PCR of markers expressed in differentiated 5T4-positive MESC ES cells purified using MidiMACS LS columns. See Table 1 for markers used. (d) Cell surface expression of 5T4 in MESC ES cells (i) grown in serum known to support undifferentiated growth and (ii) 1st passage in serum known to induce differentiation. 5T4 expression (open population) control rat IgG (closed population). (e) 5T4 cell surface expression in MESC ES cells grown for 1 passage on primary embryonic fibroblasts (i) known to support undifferentiated growth and (ii) unable to support undifferentiated growth. 5T4 expression (open population) control rat IgG (closed population).

Table 3. Common markers of ES cell integrity and differentiation

Common markers used for determination of ES cell integrity and differentiation. Abbreviations: ecto-ectoderm; meso-mesoderm; endo-endoderm; Oct-3/4, octamer binding protein-3/4; Rex-1, reduced expression-1; SSEA-1, stage-specific embryonic antigen-1; Fgf-5, fibroblast growth factor-5; ZG- ζ-globin; Bmp-2, bone morphogenic protein-2; Bmp-4, bone morphogenic protein-4; T-Bra, brachyury; Flk-1, vascular endothelial growth factor receptor-2 (VEGFR-2); K-18, keratin-18; NF-68, neurofilament-68k; Vim-vimentin; AFP, α-fetoprotein.

The invention is further described below, for the purposes of illustration only, in the following examples.

EXAMPLES

EXAMPLE 1 - Generation of m5T4 specific antibodies and m5T4-expressing cell lines

Materials and Methods

5T4-Fc Fusion Proteins

A 1004 bp cDNA fragment encoding the extracellular domain of mouse 5T4 antigen was generated by PCR and cloned by restriction digestion into the signal-pIg plus expression vector (Ingenious, R&D systems). Stable expression in Cos–7 cells (Shaw et al. (2000)) was achieved by selection in G-418 at 1mg/ml. Mouse and human 5T4-Fc fusion proteins were fractionated from tissue culture supernatant by ammonium sulphate precipitation and purified by wheatgerm agglutinin and protein G affinity chromatography. The concentration was determined by anti-human Fc-capture ELISA (Shaw et al. (2000)) and modified Bradford assay (Bradford (1976)).

Purity was assessed by silver stained SDS-PAGE. The Fc domain of m5T4-Fc was removed by overnight digestion with factor Xa protease (Roche). M5T4 extracellular domains (m5T4ex) were then enriched by negative selection on a protein G column and concentrated by centrifugal spin filter (Shaw et al. (2002)).

ELISA

Plates were coated with 50µl of antigen at 1µg/ml in 0.1M sodium carbonate buffer pH 9.3 overnight at 4°C. Plates were washed with PBST three times between each layer. Non-specific binding sites were blocked with 5% milk powder in PBST for 1 hour at 37°C. Plates were incubated successively for 1 hour at 37°C with 50µl per well of each of the following; test sample, biotinylated mouse anti rat κ/λ (1:3000 Sigma) and streptavidin HRP (1:6000 Dako). Reactions were developed with 100µl of tetra-methyl benzidine at 0.1mg/ml in 50mM citrate

phosphate buffer pH5.5, stopped by the addition of 50µl of 1M sulphuric acid and read at 450-650nm.

Polyclonal Antisera

Rabbits were immunised subcutaneously with 100µg of purified m5T4-Fc in Freunds complete adjuvant and boosted on a fortnightly regime using Freunds incomplete adjuvant. Anti-m5T4 activity was assessed by ELISA-based assay against m5T4ex on alternate weeks. Upon acquisition of significant anti-m5T4ex activity, rabbits were terminally bled by cardiac puncture, serum harvested, aliquoted and stored at –20°C.

Cell Culture

Non-adherent cells were grown in RPMI 1640 and adherent cells in DMEM (Sigma) supplemented with 2mM L-glutamine and 10% FCS; transfected cell lines were maintained under selection with 1mg/ml of G-418. Cells were maintained in a humidified atmosphere of 5% CO_2/air at 37°C and passaged on reaching 90% confluence. Four-day conditioned medium was prepared from confluent cultures of Y3Ag1.2.3. Fusion media comprised RPMI supplemented to 20% FCS, 50% conditioned medium, 2mM L-glutamine, 2mM sodium pyruvate and 1x DMEM non-essential amino acids (Sigma). Hybridoma cloning was performed in fusion media supplemented with 10ng/ml human epidermal growth factor.

Flow Cytometry

Adherent cells were removed from flasks with trypsin and washed three times at 4°C with FACS buffer: PBS plus 0.1% BSA and 0.1% sodium azide. 10^5 cell aliquots were transferred to a 96 well v-bottom plate, pelleted by centrifugation and the supernatant aspirated. All subsequent steps were incubated on ice for 30 minutes and cells washed three times with FACS buffer between layers. Tissue culture supernatants were tested neat and purified antibodies at 10µg/ml. Rat and mouse immunoglobulins were detected with rabbit anti-rat or mouse FITC direct conjugate respectively (1:30, Dako). Prior to analysis cells were fixed for 10 minutes at 4°C by the addition of an equal volume of 3.7% paraformaldehyde in PBS.

Cell Lines

A9 fibroblastic cells expressing human 5T4 (Carsberg et al. (1995)) or chimeric human-mouse (hm) and mouse-human (mh) 5T4 were generated as previously described (Shaw et al. (2002)). Lipofectamine was used to transfect A9 cells with m5T4 cDNA in pCMVα. Bulk cultures were grown for two weeks with G-418 at 1mg/ml and then assessed for 5 m5T4 antigen expression with the Rabαm5T4 antisera by flow cytometry. Positive cultures were cloned by limiting dilution, assessed for m5T4 antigen expression as before and positive wells re-cloned. The murine melanoma B16 F10 was transfected by electroporation with human or mouse 5T4 cDNA in pCMVα. Stable expression was achieved by the addition of G-418 at 1mg/ml and clones were established following two rounds of limiting dilution.

Recombinant m5T4 Vaccinia Western Reserve

The full-length m5T4 cDNA (King et al. (1999)) was cloned into the Vaccinia transfer plasmid pSC65 (Chakrabati et al. (1997)) such that it is under the control of the synthetic early promoter. Plasmid SC65-m5T4 was recombined into the tk locus of the WR strain of vaccinia virus using techniques previously described (Carroll et al. (1998)). Virus stocks were prepared in BSC-1 cells using protocols similar to that described by Earl et al (Earl et al. (1998)).

Immunisation

LOU Rats (Harlan) were immunised twice intra-muscularly with 10^8 PFU rVV-m5T4 at four-week intervals and test bled two weeks later. Four weeks after test bleeds were taken, 10^8 syngeneic splenocytes were infected overnight with rVV-m5T4 at a multiplicity of infection of 2 and used to boost the highest responder. On day four post boost this animal was terminally bled and splenectomised.

Fusion

Cell fusion was performed by the polyethylene glycol method as previously described (Kohler et al. (1976)). Fused plasmablasts were plated at a density of 10^6/ml in 96 well plates (100µl per well). After 24hrs in culture 100µl of fusion medium containing 2x HAT (Sigma) was

added. The cells were fed at days 4, 7 and 12 by 50% change of 1xHAT medium and on day 14 weaned into HT medium. At day 21, tissue culture supernatant was removed from wells positive for growth and assayed for anti-m5T4 activity by flow cytometry versus B16 F10-m5T4 or B16 F10-Neo control plasmid transfected cells and by ELISA versus m5T4-Fc fusion protein.

Positive wells were cloned four times by limiting dilution and re-screened as before. Isolated anti-m5T4 antibody isotypes were determined with a rat monoclonal antibody isotyping kit according to the instructions of the manufacturer (The Binding Site).

Antibody Production

Clarified tissue culture supernatant was brought to 45% ammonium sulphate and stirred overnight at 4°C. The precipitate was pelleted, resuspended in PBS to 10% of the original volume and dialysed at 4°C against five changes of 100 volumes of PBS. The immunoglobulin was purified by protein G affinity chromatography and the purified antibody extensively dialysed against PBS.

Immunoprecipitation, SDS-PAGE and Western Blotting

Cells were lysed at 10^7 per ml in PBS 0.5% NP40 containing 1x Complete protease inhibitors (Roche). Lysates were pre-cleared at 4°C for four hours with 5µg of control rat IgG1. Proteins coupled to rat IgG1 were complexed with 50µl of a 50% suspension of Protein G coupled Sepharose (Amersham Biosciences) and removed by centrifugation (1000g 1min).

Immunoprecipitations were performed with 5µg of test antibody, and 50µl of a 50% suspension of protein-G Sepharose. Immunoprecipitates were washed five times with lysis buffer, resuspended in 50µl of 1X SDS-PAGE sample buffer and boiled for 3 minutes. Samples were separated by SDS-PAGE using an Atto minigel system according to methods of Laemmli (Laemelli (1970)). Proteins were transferred electrophoretically to nitrocellulose with a Biorad Transblot semidry transfer system and blocked overnight at 4°C in PBST containing 5% milk powder.

All antibodies were applied for 1hr at room temperature with agitation and blots washed 5 times for 5 minutes between layers (rat IgG1 and 9A7 (10μg/ml), rabbit anti rat-HRP (1:2000 Dako) and streptavidin-Horseradish peroxidase (1:6000 Dako). Antibody binding was detected by chemiluminesence (Amersham Biosciences) according to the instructions of the manufacturer.

Immunofluorescence Microscopy

10^4 Cells were seeded onto acid washed 16mm glass coverslips in α-MEM containing 1% FCS and grown for 48hrs. Cells were washed three times with FACs buffer and fixed with 3.7% paraformaldehyde in PBS for 15mins prior to labelling or labelled at 4°C in FACs buffer, washed and then fixed. Antibodies were applied as follows; 9A7 (10μg/ml), MAb5T4 (5μg/ml), rat IgG1 (10μg/ml) or mIgG (5μg/ml) and the second layer rabbit anti-rat or mouse-FITC conjugate (1:30 Dako as appropriate) for 30mins. Non-fixed samples were then washed and fixed as described previously. Samples were mounted in PBS containing 80% glycerol and 2% 1,4-Diazabicyclo[2.2.2]octane, and sealed with clear nail lacquer.

To investigate effect of cytoskeletal disruption upon 5T4 distribution, samples were incubated with 10μg/ml of either demecolcine or cytochalasin D for two hours prior to labelling (Carsberg et al. (1995)).

Cell Attachment

Aliquots of $3x10^5$ cells were seeded in α.MEM containing 0%, 1%, or 5% FCS in each well of a 6 well plates and incubated for 24hr. Wells were washed three times with PBS to remove non-adherent cells and adherent cells trypsinised and counted by haemocytometer.

The effect of extracellular matrix proteins upon cell attachment was assessed in 96 well plates. Each well was coated with 10μg of laminin, fibronectin collagen IV or matrigel in PBS overnight at 4°C. Plates were washed 3 times with PBS and 10^3 cells seeded per well in 100μl of serum free α.MEM containing 25μg/ml transferrin (Sigma). Plates were incubated for 24hrs, washed 3 times with PBS and stained with 0.01% Crystal Violet in PBS for 15minutes. Excess dye was removed by extensive washing, plates air dried and residual dye dissolved by

agitation for 30 minutes at room temperature with 100µl per well of 10% acetic acid. The optical density was then read at 570nm.

Proliferation

Proliferation assays were performed as described (Carsberg et al. (1995); Carsberg et al. (1996)). Briefly, 10^4 cells were seeded in duplicate in 6 well plates in DMEM containing 10% FCS. 24 hours later the cells were washed three times and the medium replaced with α-MEM containing 0.5%, 1% or 5% FCS. Cells were trypsinised and absolute numbers determined on at 24-hour intervals with a Coulter counter.

Motility and Invasion Assay

Motility and invasion assays were performed as previously described (Carsberg et al. (1995); Carsberg et al. (1996)). Falcon cell culture inserts with a non-coated 8µm porous polyethylene teraphthalate membrane were used for motility assays, and coated with 10µg of Biocoat Matrigel for invasion assays (Beckton Dickinson). α-MEM containing 0.25% FCS, used for all assays, was conditioned by incubation with NIH 3T3 fibroblasts for 2 hours. 0.5 ml of conditioned medium was placed in the lower compartment and 10^4 cells seeded in 250µl of non conditioned medium in the upper compartment in multiples of four. Twenty-four hours later wells were washed and fixed with 3.7% paraformaldehyde in PBS for 20 minutes.

Migration to the lower chamber was assessed by removal of cells from the upper chamber of membranes (with a cotton bud) and comparison to the total number of cells remaining on both surfaces. Cells were stained with 0.01% crystal violet and then processed as for cell attachment.

Immunohistochemistry

Murine tissues examined were obtained in triplicate from both male and female mice. These included adult heart, lung, liver spleen, kidney, large intestine, small intestine, brain, testes, ovary and 17.5day placenta.

Immunohistochemistry was performed on 5μm cryostat sections of snap frozen tissues. Slides were fixed at room temperature for five minutes in acetone and air dried prior to re-hydration in tris buffered saline (TBS: 50mM tris pH7.6 140mM NaCl). Endogenous peroxidase activity was blocked by incubation in TBS containing 0.1% sodium azide and 0.1% hydrogen peroxide, at room temperature for ten minutes. The sections were blocked with 10% normal rabbit serum for 30 minutes, all subsequent steps were in TBS containing 1% normal rabbit serum and incubated for 30 minutes at 30°C.

Sections were stained with either 9A7 or a rat IgG1 at 10μg/ml followed by the secondary antibody, rabbit anti-rat HRP direct conjugate (1:100 Dako). Anti-mouse immunoglobulin activity in the secondary antibody was neutralised by the addition of 10% mouse serum. Immediately prior to use, reagents were spun at 4°C for 30 minutes at 13,000rpm in a bench top microfuge. Antibody labelling was visualised with di-amino benzidine and slides counter-stained, cleared, fixed and mounted as described by Southall et al (1990).

Polyclonal Rabbit Anti-Mouse 5T4-Fc

To facilitate cloning and preliminary characterisation of m5T4 transfected cell lines, a rabbit antiserum was raised against a fusion protein of the extracellular domain of mouse 5T4 fused to human IgG-Fc (m5T4-Fc). The fourth test bleed from this rabbit showed significant anti-m5T4 activity by ELISA and after boosting, the rabbit was terminally bled and the serum harvested. The resulting antiserum (Rabαm5T4) had a titre of 1:5000 by ELISA for the extracellular domain of m5T4 (data not shown).

The rabbit pre-immune serum showed no activity versus control or m5T4 transfected cells by flow cytometry (Figure 1). However, the Rabαm5T4 antiserum labelled pCMVα m5T4 cDNA transfected B16 F10-m5T4 melanoma cells and A9-m5T4 fibroblasts, but did not label control plasmid transfected A9H12 cells or h5T4 cDNA transfected A9 fibroblasts (Figure 1).

The binding of Rabαm5T4 to m5T4-Fc or B16 F10-m5T4 cells, as measured by ELISA and flow cytometry respectively, was inhibited by pre-incubation with the m5T4-Fc fusion protein (Figure 1). This effect was titratable and could not be replicated with either hIgG or h5T4-Fc (data not shown). These results establish the specificity of Rabαm5T4 antiserum for m5T4 by

ELISA and flow cytometry (1:300 dilution) and of the expression of m5T4 molecules on the transfected B16 melanoma and A9 fibroblast cell lines.

Although specific at the cell surface, immunohistochemical analysis with Rabαm5T4 showed widespread and non-specific staining of mouse placental and liver sections (data not shown). These reactivities could not be removed by exhaustive absorption with normal liver tissue and m5T4 specific antibodies proved impossible to purify by affinity chromatography. For these reasons monoclonal rat anti-m5T4 antibodies were generated.

Generation of m5T4 positive cell lines

The establishment of mouse cell lines, which showed stable m5T4 expression, was not straightforward. In the A9 cells, flow cytometric analysis showed stable expression of the m5T4-antigen over 20-25 passages. However, after passage 25 the cells began to show evidence of reduced levels of m5T4 in the population, decreased attachment, reduced proliferation after passage and failure to propagate.

These problems were not encountered during the generation of other A9 transfected cell lines expressing human or chimeric 5T4 molecules. Similarly, B16 F10-h5T4 positive cells were relatively easy to produce and maintain whilst B16 F10-m5T4 cell lines required exhaustive selection to produce cells with stable expression and behaviour in vitro. However, as the B16 F10-m5T4 cell line showed uniform growth properties and stable expression of m5T4 in culture, it was used to screen hybridoma fusions for rat anti-m5T4 antibodies by flow cytometry.

Monoclonal Antibody Isolation and Characterisation

Rats were immunised with a recombinant strain of Vaccinia Western Reserve, which encoded m5T4 (rVV-m5T4) and provided antigen expression in the context of a strong adjuvant effect. Two weeks post boost, tail bleeds showed titres of 1:3000 against m5T4-Fc by ELISA with no cross reactivity towards h5T4-Fc or hIgG (data not shown). Test sera specifically stained m5T4 transfected cells by flow cytometry and could only be blocked from doing so by pre-incubation with m5T4-Fc (data not shown).

The best responder was boosted and the resultant plasmablasts harvested and fused with the Y3 Ag1.2.3 partner cell line. Of the 960 plated wells, 151 were positive for growth and 104 of these contained rat antibodies, three of which reacted specifically with the m5T4-Fc fusion protein by ELISA. These wells were designated as 8C7, 9A7 and 10F4 by location. However, flow cytometric analysis with the B16 F10-m5T4 cell line, showed that only 9A7 reacted and therefore further analysis was limited to this antibody.

9A7 activity was specific for A9 cell lines transfected with the m5T4 cDNA and did not react with A9 cell lines transfected with either neomycin control plasmid (A9H12) or h5T4 cDNA (Figure 2).

Antibody labelling could be titrated and was inhibited by pre-incubation with a five fold molar excess of m5T4-Fc (Figure 2). Similar results were seen for B16 transfected cells (data not shown). By ELISA, 9A7 only recognised m5T4 as antigen and this recognition could be specifically inhibited by simultaneous incubation with a five fold molar excess of m5T4-Fc (Figure 3). The inhibition of 9A7 binding to m5T4-Fc was titratable and was not affected by either hIgG or h5T4-Fc. Together, these results confirm the specificity of 9A7 for m5T4 antigen.

Epitope Mapping

Chimeric A9-5T4 cell lines (mh/hm -Figure 4) were used to map the 9A7 epitope to a specific region of the mouse 5T4 molecule. Flow cytometric analysis showed that the 9A7 and MAb5T4 antibodies labelled the A9-hm5T4 and A9-mh5T4 chimeras respectively, in a non-reciprocal fashion (Figure 4). Therefore, both these cell lines expressed antigenically competent chimeric 5T4 molecules. These results localised the MAb5T4 and 9A7 epitopes to the membrane proximal regions of the human and mouse 5T4 molecule respectively.

Western Blotting and Immunoprecipitation

Reduced and non-reduced Western blots of the mouse and human 5T4-Fc fusion proteins were probed with either 9A7 or a polyclonal rat anti-m5T4 (Ratαm5T4 –Figure 5). Ratαm5T4 reacted specifically with both reduced and non-reduced m5T4-Fc (Figure 5Ai-ii).

However, the 9A7 antibody was only specific for m5T4-Fc under non-reducing conditions giving a small but significant signal with reduced h5T4-Fc (Figure 5Aii). By comparison, the detection of full-length m5T4 antigen, by Western blotting of m5T4 transfected cell lysates with 9A7 is relatively insensitive. However, partial purification of membrane glycoproteins by wheatgerm agglutinin enrichment from transfected A9 cell-lysates, reveals a broad 72 kDa band specific to the m5T4 cDNA transfected cells (data not shown).

To corroborate this data, non-reduced Western blots of 9A7 immunoprecipitates from A9-m5T4 cell lysates were probed with the Rabαm5T4 antiserum. As this antiserum cross-reacts with full length human 5T4 (Figure 5Bi), it can be used to determine the specificity of 9A7 immunoprecipitation reactions for human or mouse 5T4 molecules. The resultant 72 kDa band was only present in m5T4 cell lysates indicating that 9A7 was specific for m5T4 and did not immunoprecipitate human 5T4 antigen (Figure 5Bii).

Cellular Distribution of m5T4

The A9-m5T4 and B16-m5T4 cell lines show a punctate pattern of labelling when stained with 9A7 (Figure 6), which was independent of pre- or post-fixation and therefore not due to antibody induced antigen redistribution.

Similar patterns of staining were seen by confocal microscopy for the murine mammary carcinoma derived cell lines C127I and EMT6 confirming that punctate labelling was independent of CMV immediate early promoter driven expression.

Disruption of the actin cytoskeleton with cytochalasin D led to a redistribution of punctate staining away from the periphery of the cell. This effect was not seen upon disruption of the microtubule network suggesting that the integrity of the actin cytoskeleton is an important factor in maintaining the distribution of murine 5T4 molecules (Figure 7).

Cell lines derived from murine tumours were assessed by flow cytometry for staining with 9A7 (table 1). Positive lines included, three derived from mammary tissue, a squamous lung carcinoma and a teratocarcinoma derived embryonal carcinoma. Those that did not stain with 9A7 included a fibroblastoid cell line, two melanomas, a lymphoma, two lung arcinomas, a breast carcinoma and also an embryonic stem cell line.

Patterns of Cell Growth

Under low serum conditions A9H12 fibroblasts grow as a "pavement" type monolayer with many cell-cell contacts with little space between cells (Figure 8). Transfection of h5T4 into mouse fibroblasts results in a more dendritic morphology, fewer cell-cell contacts and an increased tendency to disperse (Figure 8). The expression of m5T4 by A9 fibroblasts resulted in long spindle shaped cells compared to plasmid control transfected cells (Figure 8).

M5T4 transfected A9 cells form colonies that stack vertically and align in a parallel fashion along the axis of the spindle. This results in the formation of "fibres" that grow by extension to connect with others, after which they spread outwards to cover the remaining free surface. This was seen in many experiments, throughout the passage window and with several independently derived clones.

A9-m5T4 antigen positive cells showed reduced proliferation when compared to the A9H12 neomycin control cell line. Of the A9-h5T4, A9-m5T4 and A9H12 cell lines, only the A9H12 neomycin cell line could be maintained in serum free media with doubling time of 75 hours. Addition to the media of FCS (0.5%) allowed all cell lines to be maintained. Proliferation rates were in the order A9H12>A9-h5T4>A9-m5T4 with doubling time of 62, 120 and 146 hours respectively. Increasing the concentration of foetal calf serum to 5% did not alter this rank order, but did decrease the differences in doubling times between the lines; A9H12, A9-h5T4 and A9-m5T4 at 53, 62 and 67 hours respectively.

Transfection of the B16 and A9 murine cell lines with m5T4 resulted in a 7% reduction of forward scatter as assessed by flow cytometry (Table 2). This implies an average reduction in cell volume upon transfection of cells with autologous 5T4. This effect was not observed in A9 fibroblasts transfected with the h5T4 cDNA, the neomycin control cassettes or the hm or mh chimeric 5T4 constructs. All cultures showed good viability with homogeneous 5T4-antigen expression by flow cytometry.

Adhesion

A9 cell lines exhibit serum concentration dependant attachment to plastic (Figure 9). The degree of this effect lessened as the serum concentration was increased but the relative differences between cell lines remained. The capacity of A9-m5T4 cells to adhere to plastic shows the most pronounced sensitivity to serum concentration followed by A9-h5T4 and then A9H12.

The extracellular matrix components collagen IV, laminin and fibronectin showed little differential effect upon adhesion of cells and followed the same trend as to for adhesion to plastic (Figure 9). However, matrigel coated wells resulted in increased adhesion of all cell lines tested but did not alter their relative propensities to adhere.

Motility and Invasion

The effect of the stable expression of human and mouse 5T4 molecules on the ability of A9 cells to actively move and invade was compared that of the A9H12 neomycin control cell line. The stable expression of human or mouse 5T4 by A9 cells did not significantly alter their propensity to invade but did increase their motility threefold and sevenfold respectively (Figure 10). These experiments were repeated three times using cells of low passage number with uniform growth and 5T4 expression. The data presented is representative of these results. Interestingly, cultures of A9-m5T4 positive cells, heterogeneous in their mouse 5T4 expression and older than 25 passages, show reduced motility in comparison to homogeneous cultures of lower passage number.

Immunohistochemistry

As the human 5T4-oncofoetal antigen was identified in placental tissue, the immunohistochemical reactivity of anti-m5T4 monoclonal antibodies was assessed against frozen sections of 17.5-day mouse placenta (Figure 11). This showed that the 9A7 antibody specifically labelled placental tissue of foetal origin. Cells of the syncitio- and cytotrophoblast showed discrete staining and the amnion was also positive.

Adult tissues examined were isolated from three individual male and female adult mice. These included heart, lung, liver spleen, kidney, large intestine, small intestine, brain, testes and ovary. Limited staining of specialised subsets of cells was seen in some of these adult tissues. In order of intensity these were; the choroid plexus in the lateral ventricles of the brain (Figure 11); the outer epithelial lining of the ovary; the glandular mucosal cells of the large and small intestine; the glomeruli of the kidney; the sinusoids of the liver; and the lining of the bronchi.

Adult tissues completely negative for 9A7 staining included the spleen, testis and heart. 9A7 failed to specifically label paraformaldehyde fixed wax embedded mouse placenta.

Discussion

The production of m5T4 positive cell lines and the description of m5T4 expression in the adult mouse required the development of a specific rabbit anti-mouse 5T4-Fc polyclonal serum (Rabαm5T4). Previous observations had demonstrated the antigenic integrity of the human 5T4-Fc fusion protein with both mono and polyclonal reagents (Shaw et al (2000)). Therefore, rabbits were immunised with a m5T4-Fc fusion protein and the resultant Rabαm5T4 antiserum was shown to be specific for the m5T4 antigen at the cell surface in B16 F10 and A9 transfected cell lines. However, Rabαm5T4 could not be used for immunohistochemistry due to high levels of background labelling. Therefore, rats were immunised with a vaccinia virus encoding m5T4 antigen and a hybridoma fusion performed, which was then screened by ELISA and flow cytometry against the m5T4-Fc fusion protein and the B16 F10-m5T4 cell line respectively. Screening of this fusion resulted in the isolation of the rat anti-mouse 5T4 antibody 9A7. Here we have demonstrated its specificity for the m5T4 antigen by flow cytometry, ELISA and immunoprecipitation.

The labelling of tumour and transfected cells lines with 9A7 confirmed expression of 5T4 antigen by m5T4 mRNA positive cells (King et al. (1999)). The epitope recognised by 9A7, was shown to possess a conformational component and was mapped to the membrane proximal region of the mouse 5T4 molecule.

Expression of either mouse or human 5T4-cDNA by transfected mouse tumour cell lines increased their motility but reduced their rate of proliferation and capacity to adhere. The

magnitude of these effects was shown to be serum concentration dependent and was greater when cells were transfected with autologous 5T4-cDNA.

Finally, the 9A7 antibody was used to describe the distribution of m5T4 in adult mouse tissues by immunohistochemistry. Selection for stable growth and expression of the m5T4 antigen by murine cell lines was relatively difficult. However, the stable expression of human or chimeric 5T4 molecules by these cells was, in comparison, relatively straightforward yielding stable and long-term expression beyond 25 passages. It is possible that over expression of autologous 5T4 molecules may deliver negative effects (e.g. through proliferation rate and adhesion changes), which are more pronounced because of species-specific influences of 5T4 antigen expression.

The specificity of 9A7 for m5T4 was confirmed by direct binding and inhibition based assays in vitro (by ELISA) and at the cell surface where binding of 9A7 to m5T4 mRNA positive cells (King et al. (1999)) could only be inhibited by the m5T4-Fc fusion protein. Western blots of m5T4-Fc fusion protein show that reduction significantly lowers its antigenicity, which implies that the 9A7 epitope, like that of MAb5T4, may be conformational in nature. However, reduced Western blots of h5T4-Fc revealed a cryptic epitope within the human molecule, which can be recognised by 9A7.

As the amino acid sequences of human and murine 5T4 show over 81% identity (Myers et al. (1994)) it is likely that the 9A7 epitope, or one very similar, is present in an altered conformation within h5T4. Reduction, electrophoresis and blotting may allow this cryptic epitope to refold into a conformation that facilitates recognition by 9A7.

Western blot analysis of full-length m5T4 antigen from cell lysates was not very sensitive with 9A7 and required enrichment of membrane glycoproteins by either immunoprecipitation or wheatgerm agglutinin affinity chromatography. Western blots of such enriched cell lysates showed a broad 72 kDa band when probed with the Rabαm5T4 antiserum. These results were similar to those previously demonstrated for human 5T4 (Hole et al. (1990)) and were limited to m5T4 mRNA positive cell lysates (King et al. (1999)). As the Rabαm5T4 antiserum used to probe 9A7 immunoprecipitation reactions also detects the human 5T4 antigen by Western blotting, the lack of a 72 kDa band from h5T4 transfected cell lysates indicates that 9A7 specifically immunoprecipitated the m5T4 antigen.

The 9A7 epitope was mapped to a region of m5T4 spanning the hydrophilic domain to the plasma membrane. The MAb5T4 epitope was also shown to map to this region of human 5T4 and also shows sensitivity to reduction (Shaw et al. (2002), Hole et al. (1990)).

Both m5T4 cDNA transfected and murine tumour derived cell lines exhibited a punctate pattern of labelling with 9A7, which localised to the cell membrane. This pattern was independent of over-expression driven by the CMV immediate early promoter and not induced by antibody mediated re-organisation. However, the disruption of the actin cytoskeleton resulted in the redistribution of 9A7 staining, which is consistent with results reported for human 5T4 antigen (Carsberg et al.(1995)).

Transfection of cells with heterologous 5T4 had a pleiotrophic effect (Carsberg et al.(1995); Carsberg et al.(1996)), which was more pronounced upon transfection with autologous 5T4. The morphological, adhesive and proliferative differences between cell lines were clear under low serum conditions but became less apparent at higher FCS concentrations. However, under all FCS concentrations examined the morphology, adhesive capacity and proliferation of the A9 cell lines was always greatest for A9H12 cells followed by A9-h5T4 and then A9-m5T4. Typically, A9H12 cells show the most adhesive morphology with a "pavement" like appearance and many cell-cell contacts (Carsberg et al.(1996)), whilst A9-m5T4 cells show the least adhesive morphology with a spindle like shape and little contact with the growth support. Both the A9-m5T4 and A9-h5T4 cell lines required >0.1% FCS for growth, whereas A9H12 could be grown short term with no FCS when supplemented with transferrin. It is likely that the difference in the ability of these cells to proliferate is linked to their morphology and adhesion to the substratum.

The stable expression of human or mouse 5T4 by A9 cells did not alter their invasive capacity but there is increased motility when compared to control transfected cells. Both the A9 and B16 F10 m5T4 cDNA transfected cell lines show a reduced mean volume after transfection in comparison to neomycin control transfected cells. The human ovarian tumour cell line, Hoc-8, also shows a similar reduction in volume when overexpressing h5T4 (not shown). As the cytoplasmic and transmembrane domains of the human and mouse 5T4 molecules are completely conserved at the amino acid level, it is possible that specific interactions resulting from the extracellular domain of autologous 5T4 molecules may be involved. Mechanisms

reported to affect cell volume include, accelerated cell cycle progression (Lemoine et al, (2001)), modulation of the actin cytoskeleton (Moustakas et al. (1998)) and ion channel mediated regulation of cell hydration (Zhande et al. (1996); Scliess et al.(2000)).

The immunohistochemical distribution of m5T4 antigen in the majority of murine adult tissues and 17.5- day placenta, were consistent with those reported for human 5T4 antigen (Ali et al. (2001); Forsberg et al. (2001)). 9A7 recognised both syncitio- and cytotrophobalst in term murine placental tissue, as well as amnion. The 9A7 antibody was also shown to label discrete subsets of cells within adult murine tissues. The observation of reactivity in the choroid plexus of the lateral ventricals of the brain is novel, as is the above background signal around the sinusoids of the liver, both of which were not seen in the human immunohistochemistry. However, whilst murine brain has been shown to be positive for m5T4 mRNA, no transcripts were detected by Rnase protection in murine (King et al. (1999)).

Here we have characterised m5T4 molecules, their tissue expression and tools (antibodies, tumour cells lines) for pre-clinical mouse models relevant to studies of anti-5T4 directed immunotherapy.

EXAMPLE 2 – Expression of 5T4 in ES cells

Materials and Methods

Cell culture

ES cells were grown in Knockout DMEM (Life Technologies, UK) supplemented with 15 % serum replacement (D3, MESC and OKO160; Life Technologies) or 15 % foetal calf serum (129; Life Technologies), and sodium bicarbonate (0.12 % w/v; Sigma, Dorset, UK), L-glutamine (2 mM; Sigma), nucleosides (6 ml of the following solution/500ml DMEM: adenosine (80 mg), guanosine (85 mg), cytidine (73 mg), uridine (73 mg) and thymidine (24 mg) dissolved in 100 ml water; Sigma), 2-mercaptoethanol (50 μM; Life Technologies) and LIF (1000 units/ml of ESGRO; Clonetech, UK) at 37°C/5% CO_2. 129 (a gift from Dr. Wolfgang Breitwieser, PICR; derived from OLA mice), MESC (a gift from Dr. Rhod Elder, PICR; derived from 129/OLA mice) and D3 (American Type Culture Collection (ATCC)

CRL-1934; derived from 129/Sv+c/+p mice) ES cell lines were grown on irradiated STO fibroblast feeder layers (ATCC). OKO160 ES cell line (a gift from Dr. Austin Smith, Edinburgh, UK) was grown on gelatin-treated plates in the presence of 200 μg/ml G418 due to targeted integration of LacZ in the Oct-4 locus. The media was replenished every 24h and cells passaged before confluency (Smith (1992)). To establish ES cell clones, 129 ES cell colonies were picked, treated with trypsin for 5 minutes, replated in single wells of a gelatin-treated 96-well plate and expanded.

Differentiation of ES cells

ES cells were transferred to gelatin-coated plates for 1 day in the presence of LIF and then replenished with ES media lacking LIF. The medium was changed daily and monolayer cells passaged before confluency. Differentiation of ES cells as suspended embryoid bodies was performed by transferring undifferentiated cells to bacteriological Petri dishes and subsequent growth in LIF deficient medium. The medium was changed daily.

Fluorescent staining of ES cells

ES cells (5 x 10^6 cells/well in a 96-well plate) were incubated with rat anti-mouse 5T4 monoclonal antibody (9A7) as described above, rat anti-mouse Forssman antigen (Willison et al. (1978)) or rat control antibodies (10 μg/ml in 0.2% BSA/0.1% sodium azide in PBS) for 1 h on ice. Cells were washed 3 times and resuspended in FITC-conjugated rabbit anti-rat Ig for 1 h (1:30 dilution; DAKO, UK). Cells were washed twice as described above, fixed in 1 % formaldehyde solution and cell fluorescence measured in a Becton Dickinson FACScan.

RT-PCR

RNA was extracted from cells using RNazol B according to the manufacturer's instructions (Biogenesis, UK). RNA was treated with DNase (Promega, UK) and phenol/chloroform extracted. Synthesis of cDNA from mRNA transcripts was performed using the following method: RNA (10 μg), dNTP (250 μM), oligo dT (5.0 μg total; Promega, UK), AMV reverse transcriptase (40 units) in a total volume of 200 μL and incubated at 42 °C for 1 hour. Semi-quantitative RT-PCR of 5T4 was performed using 1 μl of the cDNA solution described above and 25-30 cycles. RT-PCR was performed using 5 μl of the cDNA solution and 35 cycles.

Since the fibroblast feeder layer contains 5T4 transcripts, MESC ES cells were grown for several passages on gelatin-treated plates to remove the fibroblast feeder cells prior to the extraction of RNA. Primers used were as follows (read 5' to 3'; forward-F, reverse-R): 5T4 F - aactgccgagtctcagatacc, R - atgataccttccatgtgatcc, 55 °C, 506bp; β-tubulin F - tcactgtgcctgaacttacc, R - ggaacatagccgtaaactgc, 55 °C, 317bp; fgf-5 F -ggcagaagtagcgcgacgtt, R - tccggttgctcggactgctt, 50 °C, 537/515bp [27]; bmp-2 F - gagatgagtgggaaaacg, R - gcagtaaaaggcatgatagc, 55 °C, 606bp; zeta globin F - gatgaagaatgagagagc, R - agtcaggatagaagacagg, 55 °C, 406bp; Oct 3/4 F - agaaggagctagaacagtttgc, R - cggttacagaaccatactcg, 55 °C, 415bp; Rex-1 F - tgaccctaaagcaagacg, R - ataagacaccacagtacacacc, 54°C, 414bp.

Western blotting of 5T4

Cells were trypsinised and incubated in gelatin-treated plates for 30 mins at 37°C/5% CO_2 to allow the fibroblast feeder layer to attach to the plate. ES cells in suspension were removed, washed and resuspended in lysis buffer (1 x 10^7 cells/ml in 0.5 M Tris, 1.5 M NaCl, 0.5 % v/v NP-40, 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) on ice for 20 min) and 20 µl of the lysate separated by unreduced SDS-PAGE. Positive and negative controls represent cell lysates of A9 cells transfected with either m5T4 cDNA or control vector respectively. Proteins were transferred onto nitrocellulose membrane using the Novoblot semi-dry transfer system (Amersham Pharmacia, UK) and the membranes blocked in 5 % milk/0.05 % Tween/PBS overnight at 4 °C. The membrane was probed using rabbit anti-m5T4 polyclonal antibody described above followed by HRP-conjugated sheep anti-rabbit immunoglobulins (DAKO, UK) and developed by enhanced chemiluminescence (Amersham Pharmacia, UK). Western blot images were captured using an Epi Chemi II Darkroom and Sensicam imager with quantification determined by Labworks 4 (UVP, CA, USA).

MACS separation of 5T4-positive MESC ES cells

MESC ES cells were grown as described above, trypsinised and washed in PBS. 5T4-positive cells were isolated using rat anti-m5T4 Ab 9A7 (10 µg/ml), goat anti-rat Ig magnetic beads and MidiMACS LS columns according to the manufacturer's instructions (Miltenyl Biotech, Surrey, UK).

RESULTS

We have used immunofluorescence with a rat monoclonal antibody (mAb) recognising m5T4 (9A7) to study cell surface expression of mouse ES cells following removal of LIF. 5T4 antigen is not detected on the surface of undifferentiated ES cells using mAb 9A7 (Figure 12a). Following withdrawal of LIF for 3 days 5T4 antigen is detected on all the ES cell lines, with the percentage of positive cells varying between 7.1 % (OKO160) and 50.0 % (MESC). Over the 12-day differentiation period there is considerable variation in both the timing of peak 5T4 antigen expression and the proportion of cells labeling positive between the cell lines. For example, MESC ES cell line exhibits peak expression around day 9 with 85.8 % of the population positive, whereas D3 ES cells exhibit a steady increase in positive cells which peaks at 43.4 % on day 12. OKO160 and 129 ES cell lines exhibit similar proportions of positive cells at day 3 (7.1 and 9.0 % respectively) and day 6 (30.6 and 34.0 % respectively) and both cell lines exhibit peak cell staining at day 9 (54.6 and 68.2 % respectively). However the proportion of OKO160 cells staining for 5T4 antigen is decreased significantly by day 12 (from 54.6 % to 17.0 %) whereas 129 is only slightly reduced (from 68.2 to 67.3 %). In all of the cell lines there is a shift in the entire population of cells staining for 5T4 antigen suggesting that it is expressed on all differentiating cells. Increase in total 5T4 protein following removal of LIF was confirmed by western blot analysis of cell lysates using a rabbit anti-m5T4 polyclonal antibody, described above, with various 5T4 isoforms apparent (Figure 12b). 5T4 is also detected on MESC ES cells differentiated as embryoid bodies for 12 days (Li et al. (2001)) (Figure 12c). These results demonstrate the use of cell-surface 5T4 oncofoetal antigen for assaying the differentiation state of mouse ES cells in a single non-destructive assay. Current differentiation-specific antigens (e.g. Flk-1) are unable to identify differentiated ES cells in a single assay since they are either transiently upregulated or expressed on a sub-population of cells, or both. Thus, the 5T4 oncofoetal antigen represents a novel cell-surface marker of mouse ES cell differentiation and lack of expression is a sensitive indicator of undifferentiated ES cell integrity.

Many ES cell techniques utilise cloning and expansion of early passage cell lines. Therefore we have assayed the effects of cloning and extended passage on the expression of the 5T4 antigen to assess its suitability as a differentiation marker for these techniques (Figure 12d, e). Undifferentiated growth of MESC ES cells for 10 passages had no effect on the level of cell-surface 5T4, detected using the 9A7 mAb (Figure 12d). Similarly, cloning of five 129 ES cell

colonies and subsequent expanded growth of the clones had no effect on cell-surface 5T4 expression (Figure 12e – a single representative colony is shown). Removal of MESC ES cells from a fibroblast feeder layer and subsequent passage on gelatin-treated plates had no effect on 5T4 antigen expression (data not shown, compare Figures 12ai and 12gi). These results demonstrate that 5T4 can be used as a differentiation marker of cloned and extended passage ES cell lines, potentially useful for a range of ES cell techniques.

The increase in 5T4 antigen on ES cells upon removal of LIF is associated with transcriptional upregulation, with both MESC and OKO160 ES cells exhibiting increased 5T4 mRNA (Figure 12f; using semi-quantitiative RT-PCR). The maximal level of 5T4 transcripts in MESC ES cells (Figure 12f i) occurs at day 3, which precedes the maximal level of protein expression (day 6; Figure 12a i). The maximal expression of transcripts in OKO160 cells occurs at day 9 (Figure 12f ii) which corresponds with maximal protein expression (Figure 12a iii). There is a clear reduction in transcripts in MESC and OKO160 cell lines after maximum protein expression. 5T4 transcripts are not detected by RT-PCR (35 cycles) in undifferentiated MESC ES cells (data not shown) suggesting that the increase in 5T4 protein upon removal of LIF is probably due to transcriptional upregulation, although increased mRNA stability cannot be discounted. Low levels of 5T4 transcripts are detected in undifferentiated OKO160 ES cells (data not shown), perhaps reflecting the slightly increased level of antigen in this cell line (Figure 12a iii). These results demonstrate that detection of 5T4 transcripts, as well as the antigen, may be useful for determining the undifferentiated and differentiated state of mouse ES cells.

To confirm that upregulation of 5T4 expression upon removal of LIF correlates with differentiation of ES cells we assayed various ES cell-specific (Oct 3/4, Rex-1, Forssman antigen) and differentiation-specific (Fgf-5, ZG and Bmp-2) markers (Figure 13a, b). These results show that upregulation of 5T4 correlates with a decrease in the ES cell-specific Forssman antigen (Figure 13b) and the detection of differentiation markers (Figure 13a), confirming that 5T4 is upregulated during the differentiation of ES cells. Most strikingly, the ES cell-associated Oct 3/4 and Rex-1 transcripts do not decrease appreciably in MESC, D3 or 129 ES cells for at least 12 days following removal of LIF. These transcripts are commonly used to confirm the presence of undifferentiated ES cells in monolayer culture (Rathjen et al. (1999)). Our results clearly show that the presence of Oct-3/4 or Rex-1 transcripts in ES cell monolayer culture is not sufficient to confirm the presence of a homogeneous undifferentiated

ES cell population. The presence of these transcripts for at least 12 days following removal of LIF may be due to either undifferentiated ES cells within the population or slow transcript turnover, or both. OKO160 ES cells have a targeted insertion in a single Oct-4 allele (A. Smith, personal communication) which is likely to account for the decrease in Oct-3/4 transcripts in this cell line, although Rex-1 transcripts are still evident 12 days following removal of LIF. There is some disparity between the differentiation markers expressed by the ES cell lines (Figure 13a). For example, Fgf-5 is transiently detected in all but 129 cells, and ZG is transiently detected in all but MESC cells (Figure 13a). This has also been observed with other differentiation markers in these ES cell lines (Ward et al, manuscript in preparation). The Forssman antigen is also limited as a sole marker of ES cell integrity since a significant proportion of cells express the antigen at least 3-days following removal of LIF (Figure 2b).

The immunofluorescent analysis of cell-surface 5T4 antigen expression shows a shift in the entire population of differentiating cells, most notably in the MESC ES cell line (Figure 12ai), suggesting the antigen is expressed on all differentiating cells. To confirm that 5T4 is expressed on cells derived from all three germ layers, MESC ES cells were assayed for the presence of cell-specific transcripts following purification of the 5T4-positive population (Figure 13c; Table 3). The presence of transcripts for AFP, NF-68 and T-Bra in the 5T4-positive cell population demonstrates the presence of endoderm, ectoderm and mesoderm cell lineages respectively (Figure 13c; Table 3). The presence of Fgf-5, Bmp-2 and -4, K-18 and Vim further confirm the presence of the three germ layers in the 5T4-positive cells.

Detection of the 5T4 oncofoetal antigen enables the optimisation of ES cell culture conditions (Figure 13d, e). For example, serum batches can be rapidly tested for their ability to maintain the undifferentiated integrity of ES cells by assaying for the presence of 5T4 (Figure 13d). This is a useful technique since current methods of serum batch testing are laborious (Smith (1992)). 5T4 antigen expression on ES cells can also be used to identify batches of primary embryonic fibroblast (PEF) feeder layers able to maintain ES cells in an undifferentiated state (Figure 13e). We have found a small number of PEF batches that are unable to maintain the undifferentiated integrity of ES cells (using morphological analysis) and this is reflected by increased 5T4 antigen expression on ES cells during the first passage on these feeder layers (Figure 13e). Importantly, upregulation of the 5T4 antigen under these adverse growth conditions precedes any morphological sign of differentiation (data not shown). Thus,

assaying ES cells for 5T4 antigen expression enables the rapid screening of culture conditions for the undifferentiated growth of the cells. Conversely, culture conditions that are designed to induce differentiation of ES cells could also be optimised using this technique. A further application of this method could include the screening and identification of cells derived from mouse blastocysts for establishing new ES cell lines (Brook et al. (1997)). Overall, non-destructive fluorescent analysis of the 5T4 oncofoetal antigen provides an assay of ES cell integrity and differentiation that can be easily performed during routine ES cell culture using a small aliquot of cells.

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All publications mentioned in the above specification, and references cited in said publications, are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

Claims

1. A method for detecting the differentiation status of stem cells comprising detecting the expression of 5T4 antigen in said stem cells.

2. A method as claimed in claim 1 wherein a low level of 5T4 antigen expression indicates undifferentiated stem cells.

3. A method as claimed in claim 2 wherein said stem cells are mammalian stem cells.

4. A method as claimed in claim 3 wherein said stem cells are embryonic stem cells.

5. A method as claimed in claim 2 or 3 wherein said stem cells are murine, human, porcine, feline or canine.

6. A method as claimed in any of claims 2 to 5 wherein said 5T4 expression is detected by anti-5T4 antibodies.

7. A method of detecting differentiation status of mammalian stem cells comprising the steps of:

a) taking a sample of stem cells;

b) incubating said sample with a labelled anti-5T4 antibody such that specific binding of anti-5T4 antibody to 5T4 antigen occurs; and

c) detecting said binding.

8. A method for separating a population of undifferentiated or differentiated mammalian stem cells from a mixture of differentiated and undifferentiated stem cells comprising:

a) binding cells with anti-5T4 antibody;

b) separating cells with bound antibody from cells with no bound antibody;

c) unbinding the antibody from the cells; and

d) isolating the cells.

9. A method as claimed in claim 8 wherein said isolated cells are viable.

10. A method for testing growth media for its use in maintaining mammalian stem cells comprising detecting expression of 5T4 comprising the steps of:

a) taking mammalian stem cells in culture;

b) applying test media; and

c) assessing 5T4 expression in the absence or presence of said media wherein the presence of 5T4 is an indication of stem cells undergoing differentiation.

11. A method for detecting the ability of a test compound to induce mammalian stem cell differentiation comprising the steps of:

a) incubating a mammalian stem cell culture in the presence or absence of said test compound;

b) detecting 5T4 expression; and

c) comparing the levels of 5T4 expression in cells wherein increased 5T4 expression in those cells incubated in the presence of said test compound indicates differentiation induction by said test compound.

12. Use of an antibody recognising 5T4 in a method of detecting differentiated mammalian cells.

13. Use of an antibody recognising 5T4 in a method of testing growth media for its use in maintaining mammalian stem cells.

14. A method for detecting differentiation status of a mammalian stem cell comprising:

a) introducing into a stem cell a vector comprising a 5T4 promoter sequence operably linked to a nucleic acid encoding a reporter gene;

b) detecting an increase in expression of the reporter gene as an indication of differentiation.

15. A method of modifying a mammalian stem cell comprising introducing a nucleic acid sequence into a mammalian cell such that said nucleic acid sequence is placed under the control of the 5T4 promoter sequence. 16. A method of modulating mammalian stem cell differentiation comprising modulating 5T4 expression or functional activity. 17. Use of an

agent that modulates 5T4 expression or functional activity in the modulation of mammalian stem cell differentiation.

18. An isolated antibody recognising the membrane proximal extracellular domain of murine 5T4.

19. An isolated antibody as claimed in claim 18 wherein said antibody is an isolated rat monoclonal anti-5T4 antibody, 9A7.

Abstract

The present invention relates to methods for detecting the differentiation status of stem cells comprising detecting the expression of 5T4 antigen in said stem cells. The present invention also relates to methods for separating populations of undifferentiated or differentiated mammalian stem cells from a mixture of differentiated and undifferentiated stem cells through detection of 5T4 expression.

Cell line	Origin	Flow Cytometry
A9 neo	Lung fibroblast L cells	-
A9-m5T4	Lung fibroblast L cells	++++
B16 F10 Neo	Melanoma	-
B16 F10-m5T4	Melanoma	++
EMT6	Mammary adrenocarcinoma	+++
C127 I	Mammary carcinoma	+++
Clone M3	Melanoma	-
EL4	Lymphoma	-
KLN-205	Squamous cell lung carcinoma	+/-
JC	Breast adenocarcinoma	-
LL/2	C57BL Lewis lung carcinoma	-
Mosec	Ovarian carcinoma*	-
Nulli 2A	Embryonal carcinoma	+
129 ES	Embryonic stem cell	-
CL-S1	BALB/c mammary pre-neoplastic alveolar nodules	+/-

Table 1.

Cell Line	Mean FSC	SD	Percentage of Neo control FSC
B16 F10-Neo	547.1	2.1	100
B16 F10-m5T4	508.9	2.1	93.00
B16 F10-h5T4	550.7	0.6	100.6
A9-H12	577.9	1.0	100
A9-m5T4	538.4	6.6	93.1
A9-h5T4	573.2	5.2	99.2
A9-mh5T4	573.4	13.6	99.2
A9-hm5T4	572.5	8.9	99.1

Table 2.

FIGURE 1

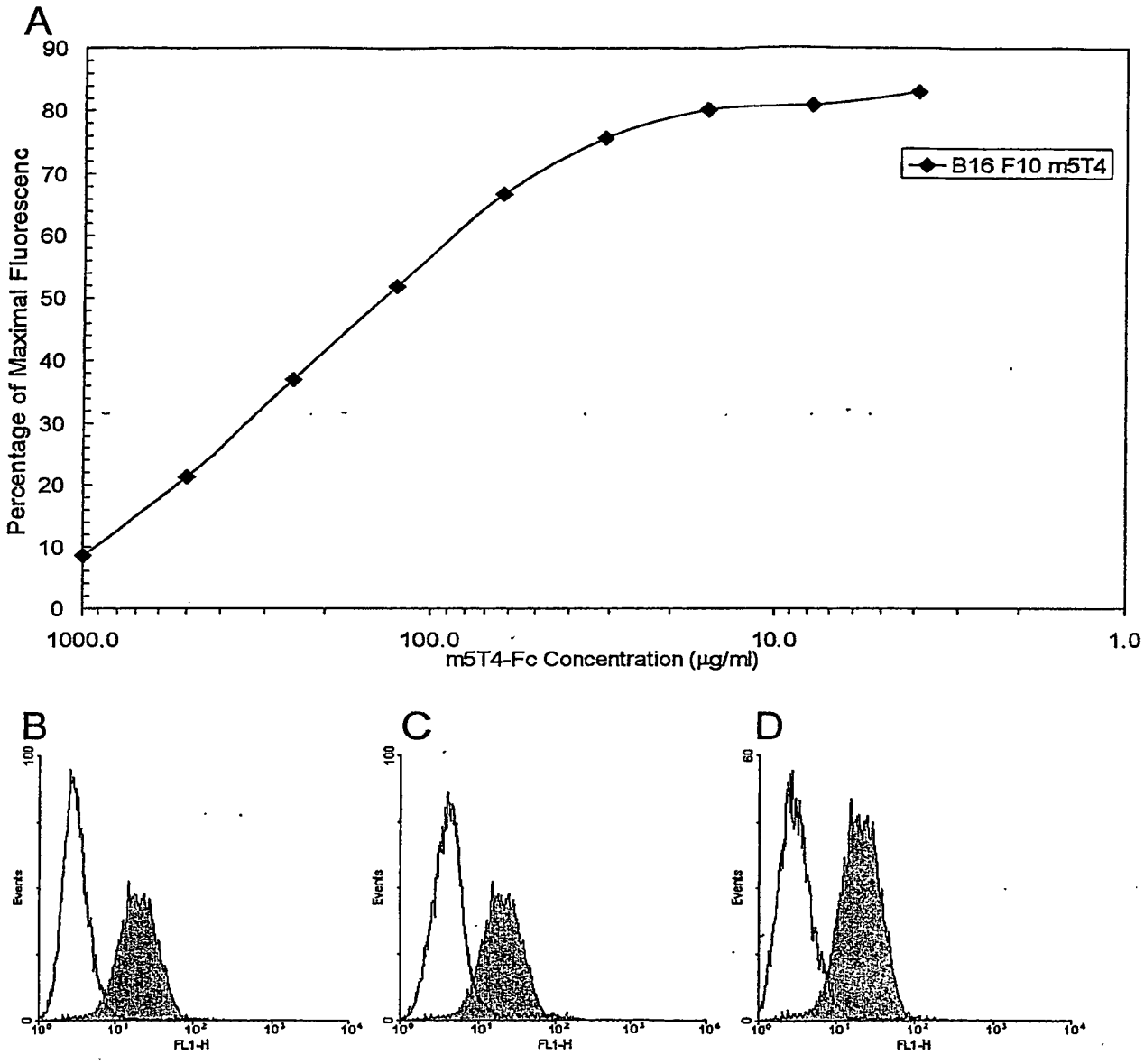

A
Percentage of Maximal Fluorescenc
90
80
70
60
50
40
30
20
10
0
1000.0
100.0
10.0
1.0
m5T4-Fc Concentration (µg/ml)
B16 F10 m5T4
B
C
D
Events
FL1-H

FIGURE 2

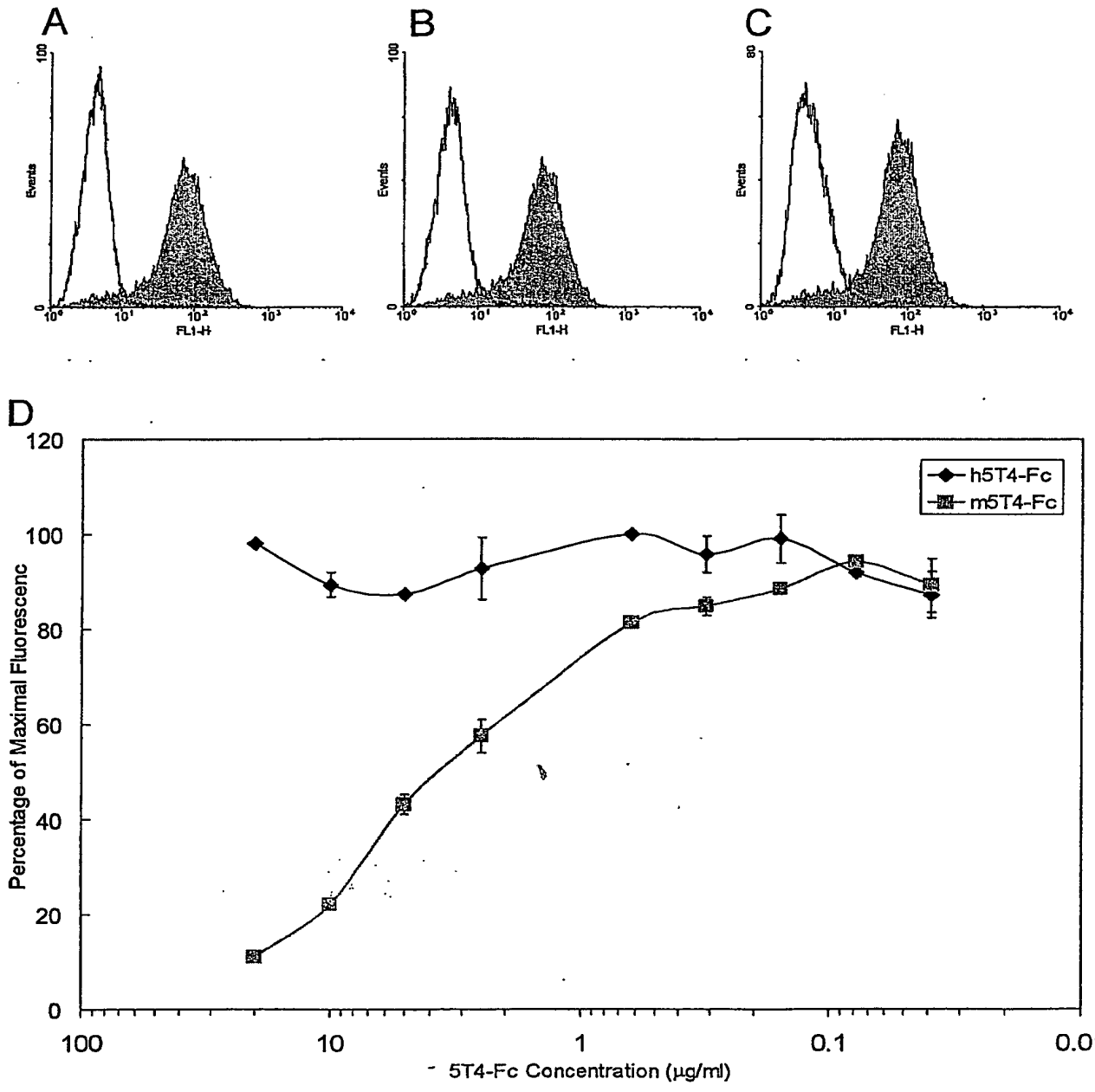

A
B
C
Events
FL1-H
D
h5T4-Fc
m5T4-Fc
Percentage of Maximal Fluorescenc
5T4-Fc Concentration (µg/ml)
120
100
80
60
40
20
0
100
10
1
0.1
0.01

FIGURE 3

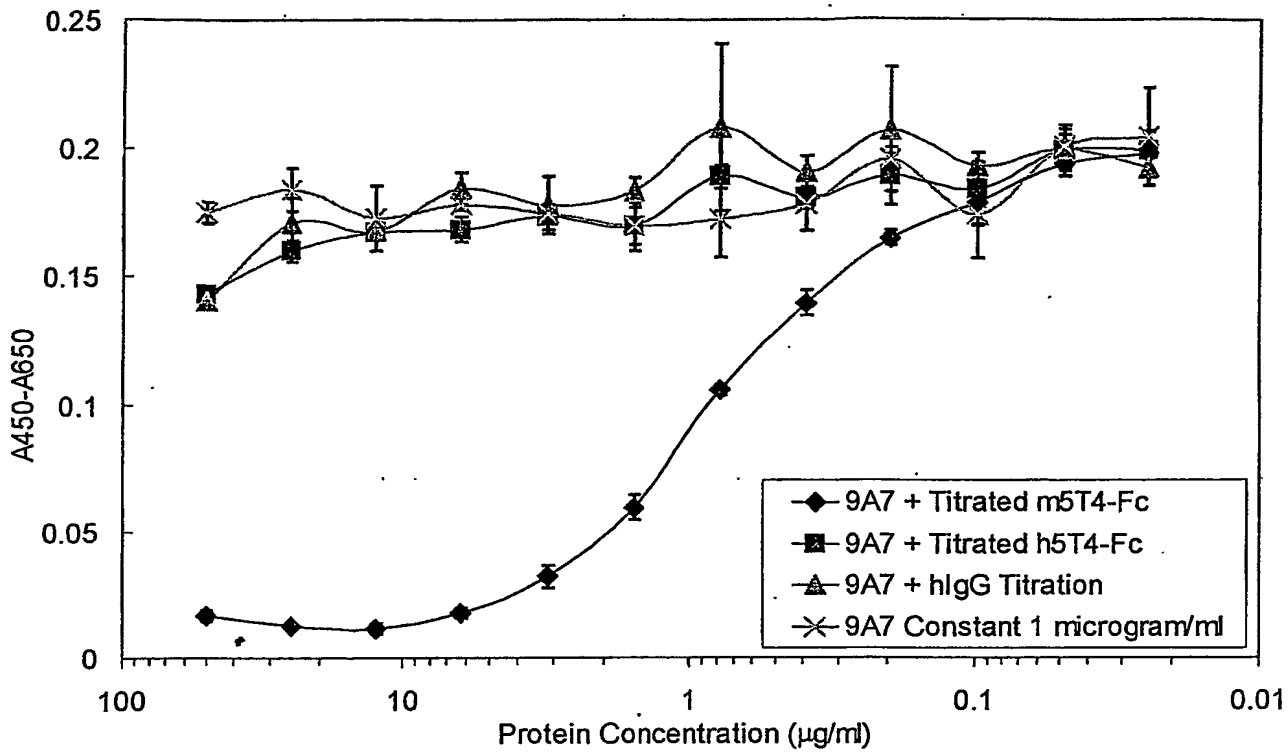
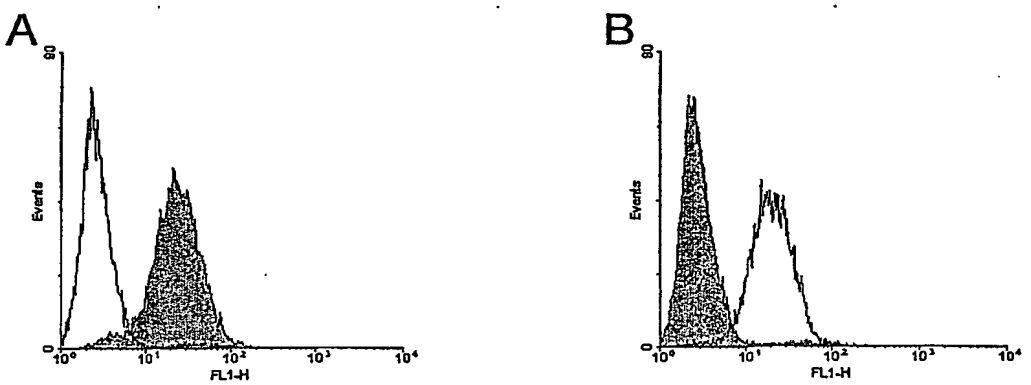

0.25
0.2
0.15
0.1
0.05
0
A450-A650
100
10
1
0.1
0.01
Protein Concentration (µg/ml)
9A7 + Titrated m5T4-Fc
9A7 + Titrated h5T4-Fc
9A7 + hIgG Titration
9A7 Constant 1 microgram/ml

FIGURE 4


A
B
Events
FL1-H

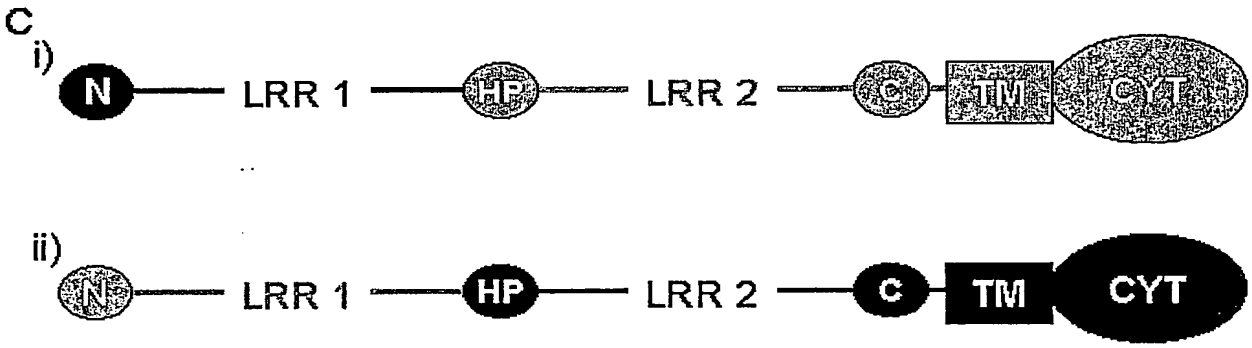
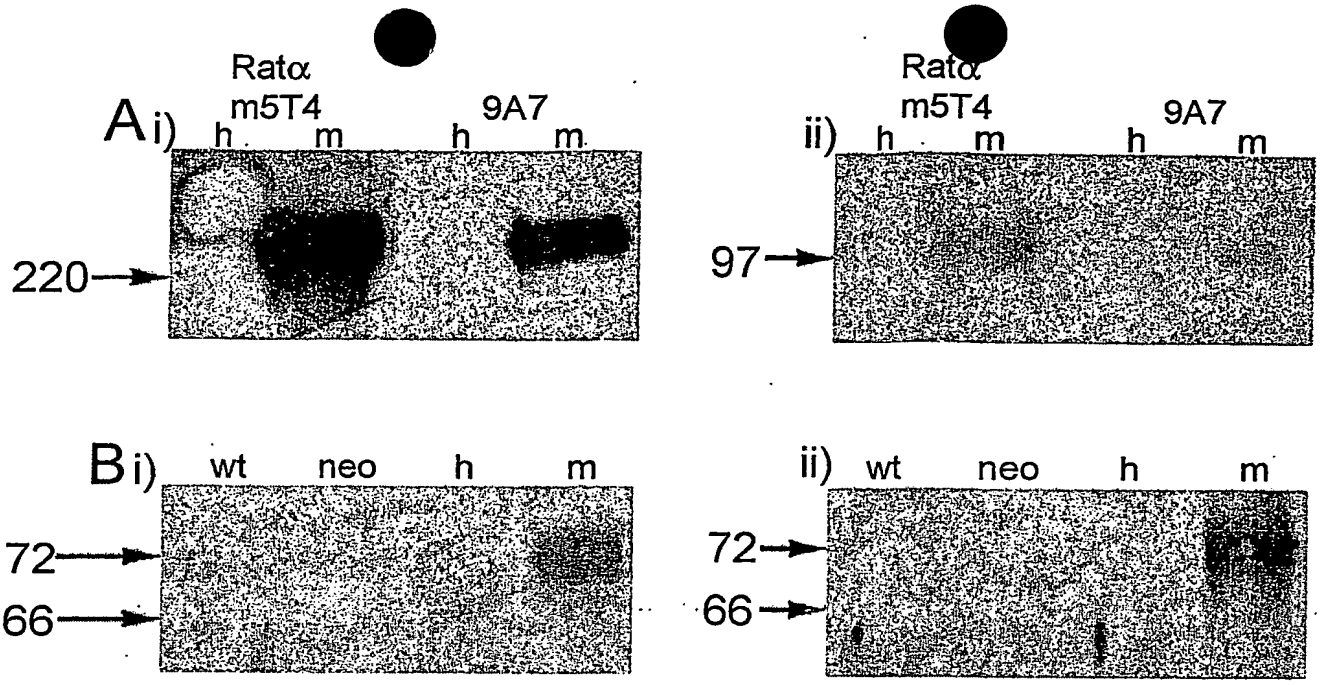
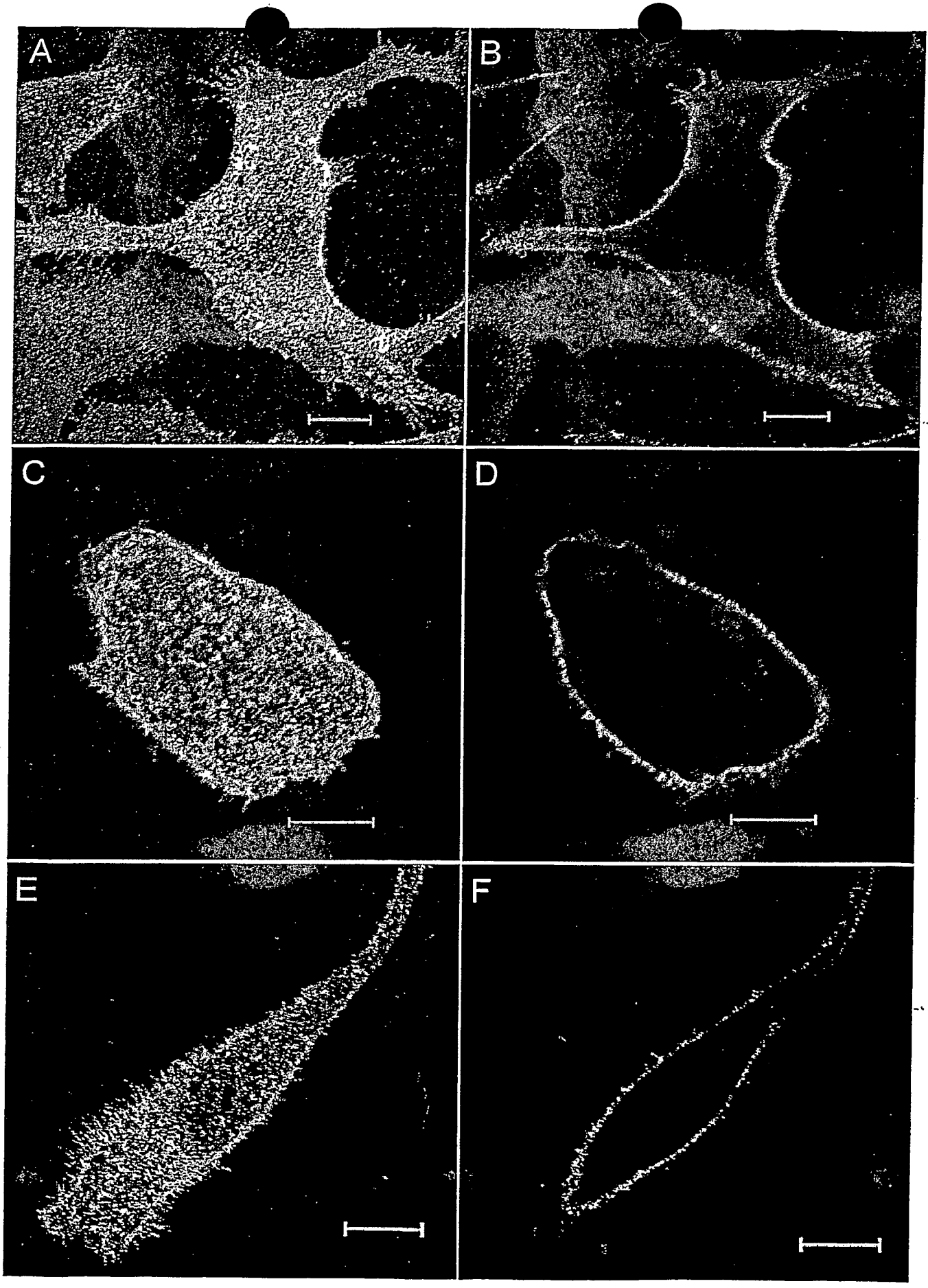
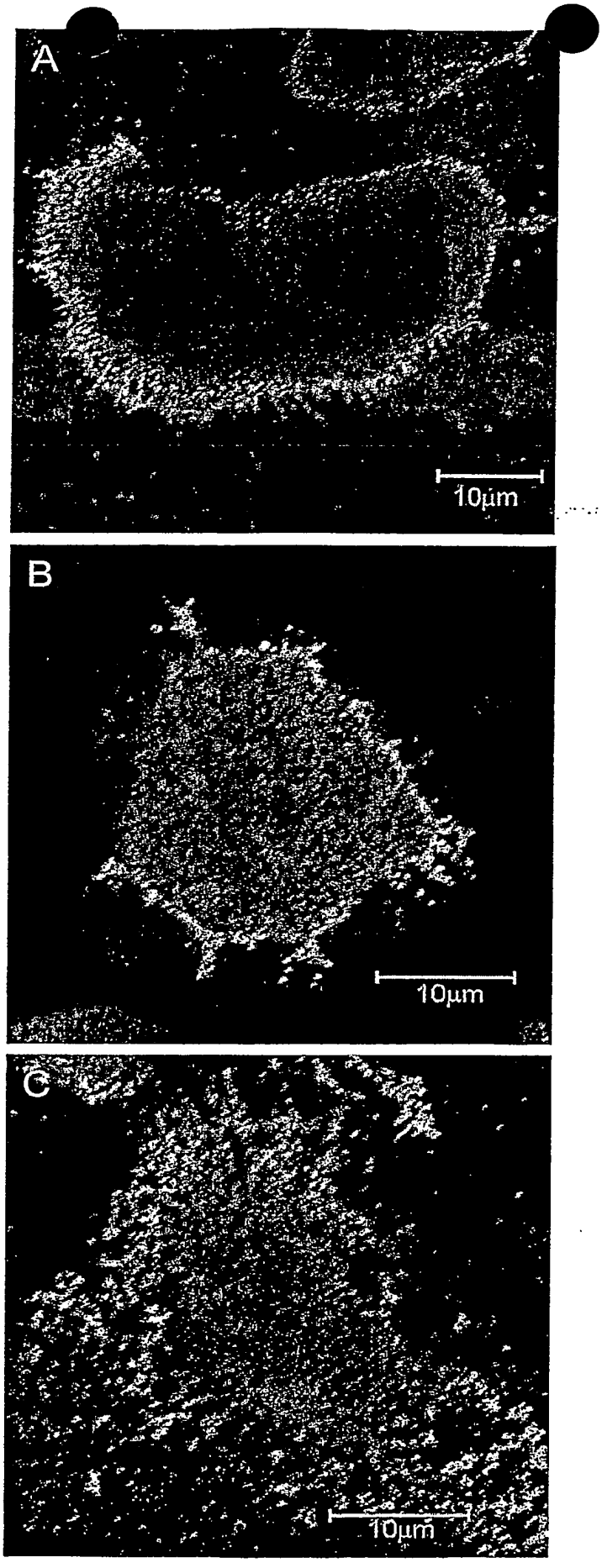

C
i)
N
LRR 1
HP
LRR 2
C
TM
CYT
ii)
N
LRR 1
HP
LRR 2
C
TM
CYT

FIGURE 5




A
B
C
D
E
F


A
10µm
B
10µm
C
10µm

FIGU. 18

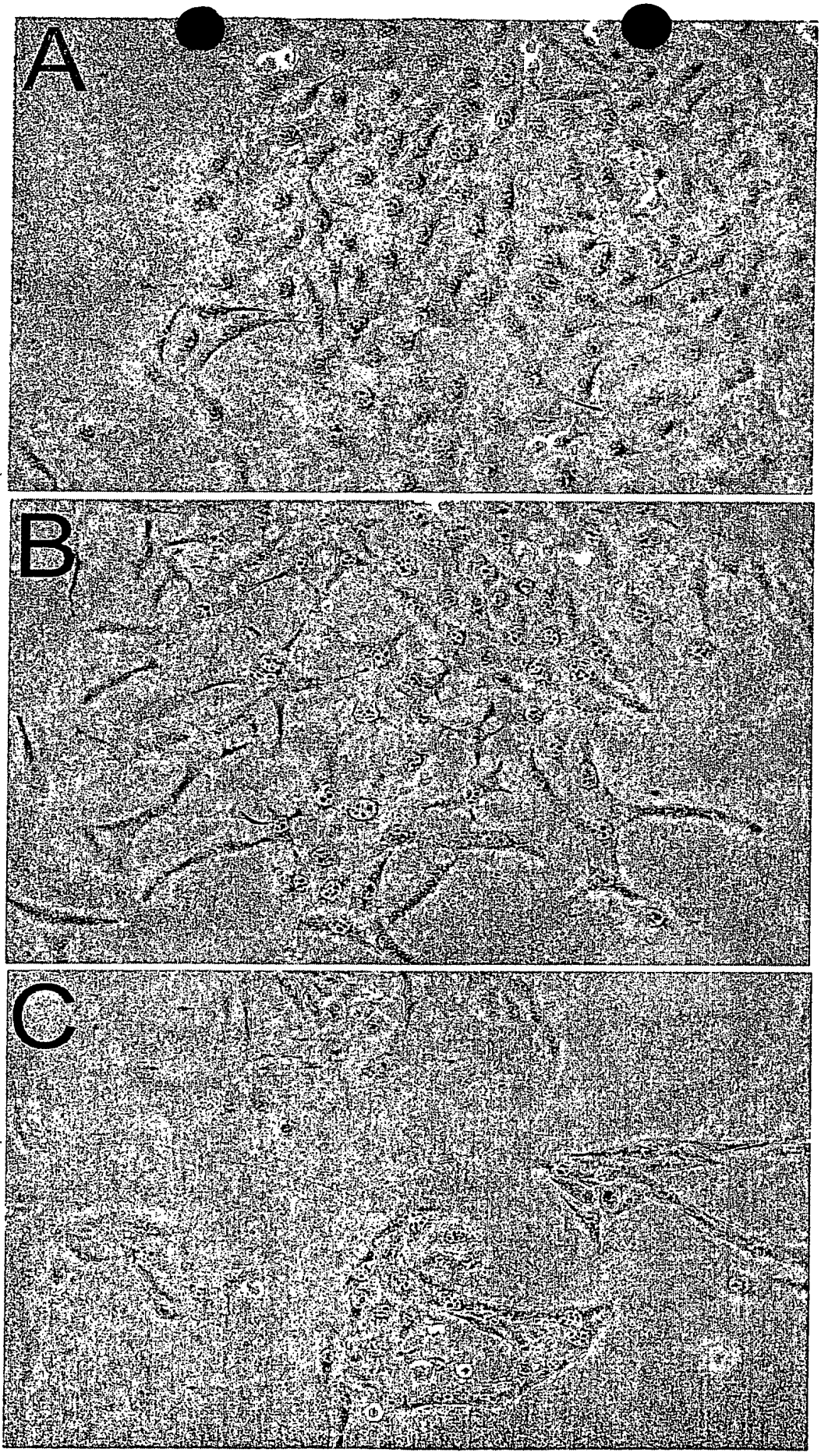
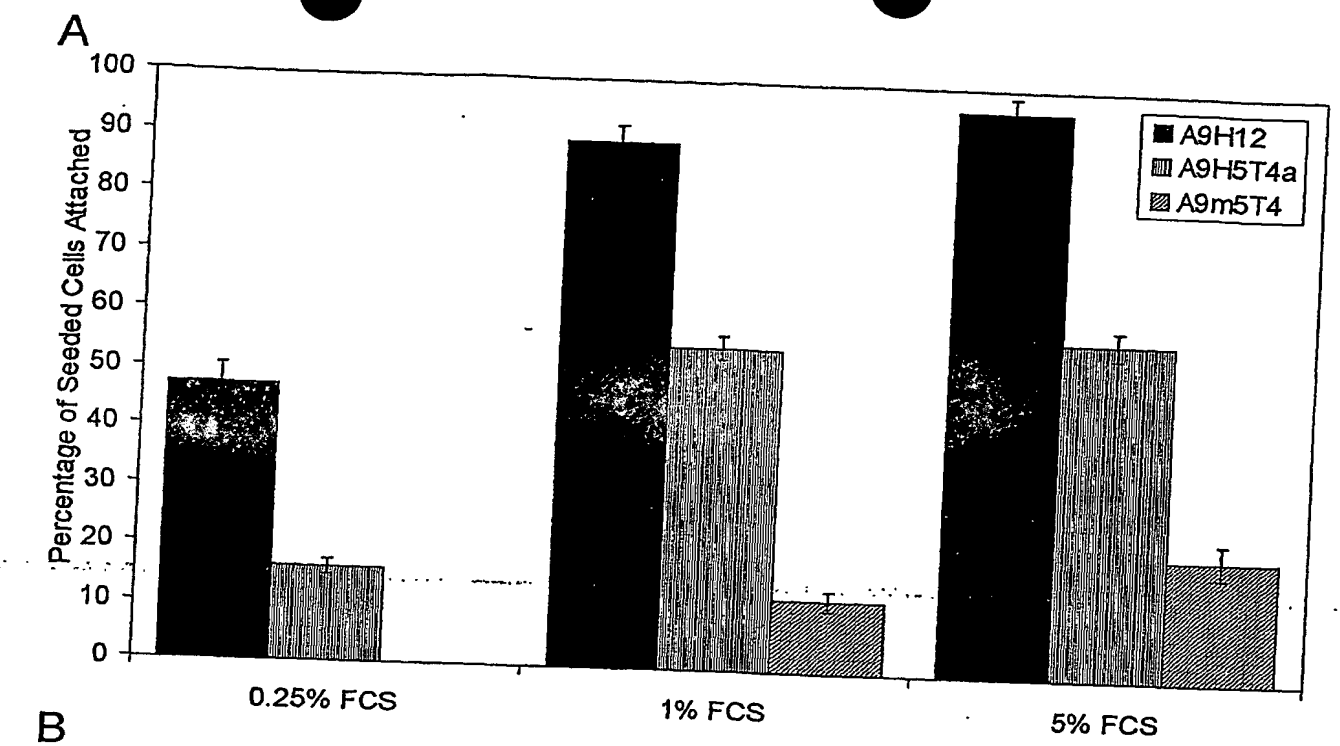


FIGURE 9


A
Percentage of Seeded Cells Attached
100
90
80
70
60
50
40
30
20
10
0
A9H12
A9H5T4a
A9m5T4
0.25% FCS
1% FCS
5% FCS

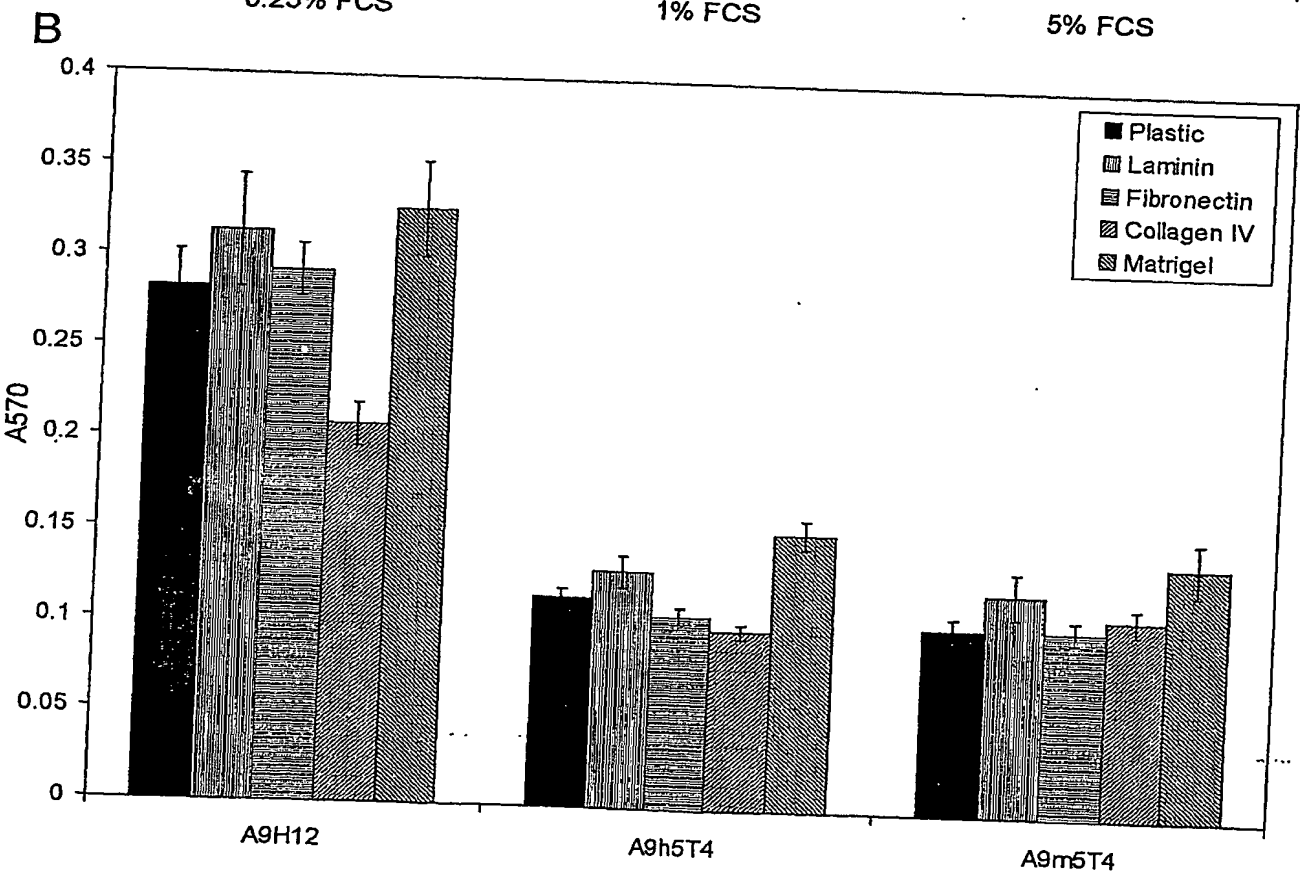
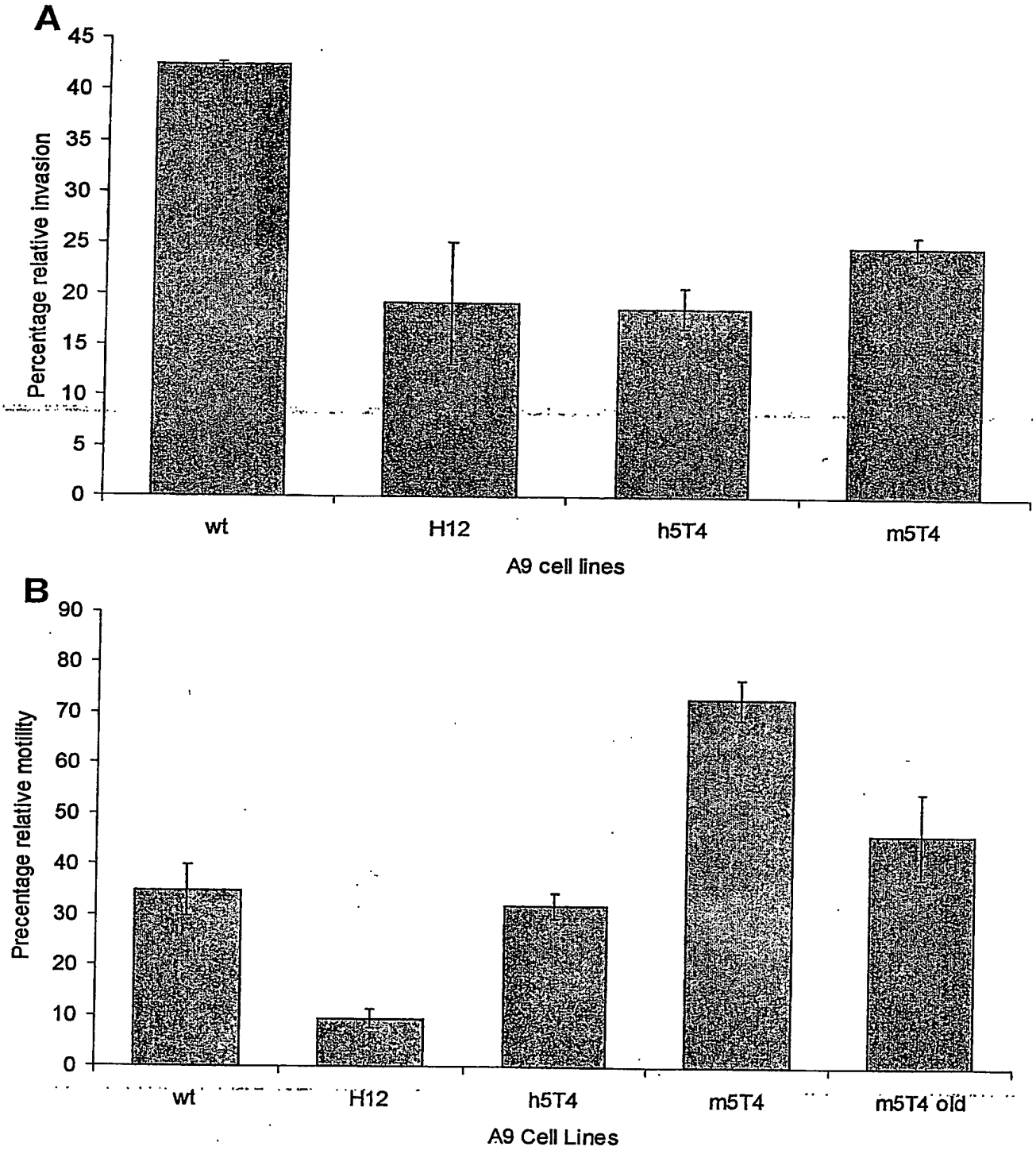

B
A570
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Plastic
Laminin
Fibronectin
Collagen IV
Matrigel
A9H12
A9h5T4
A9m5T4

FIGURE 10


A
Percentage relative invasion
45
40
35
30
25
20
15
10
5
0
wt
H12
h5T4
m5T4
A9 cell lines
B
Precentage relative motility
90
80
70
60
50
40
30
20
10
0
wt
H12
h5T4
m5T4
m5T4 old
A9 Cell Lines

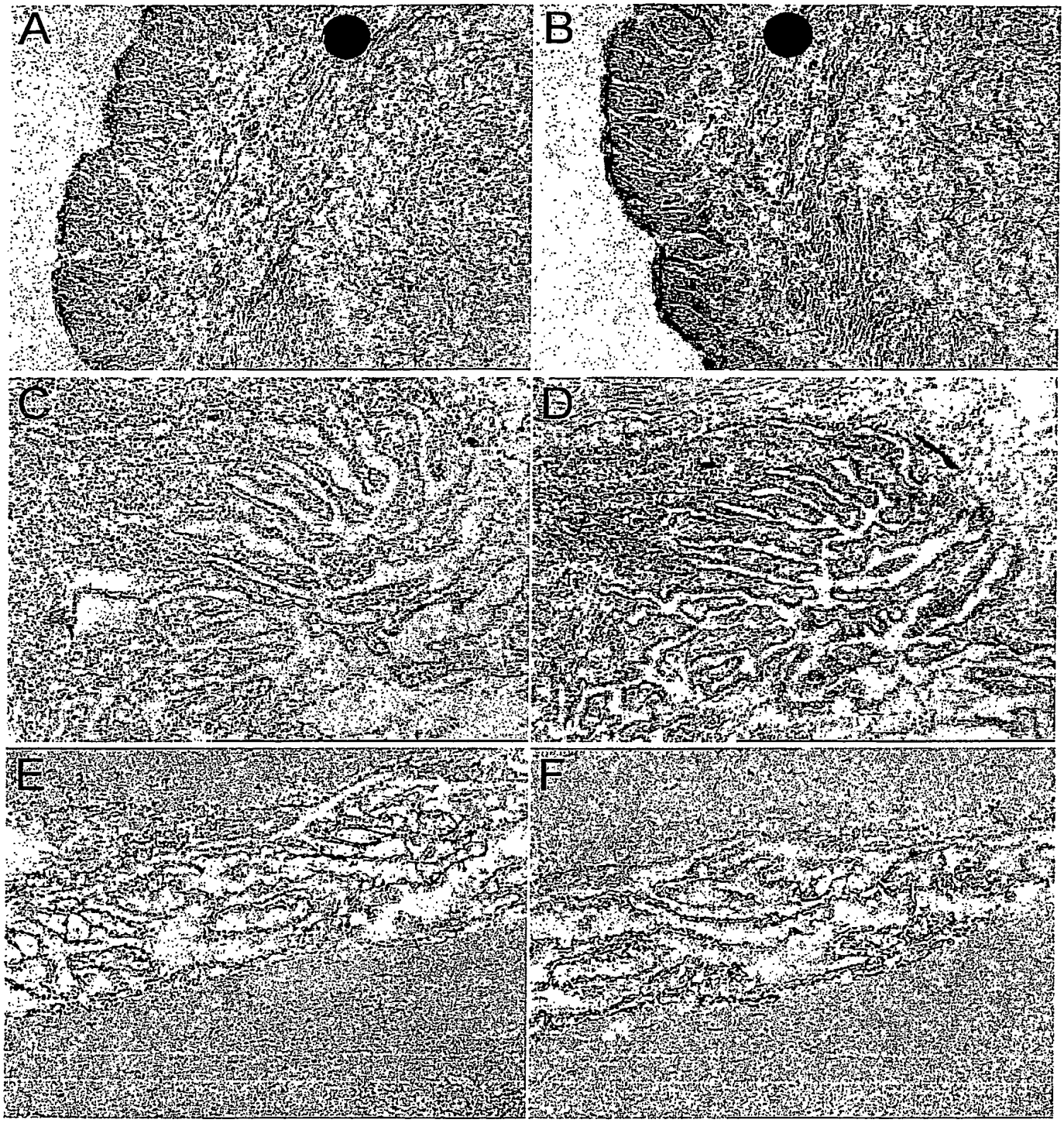
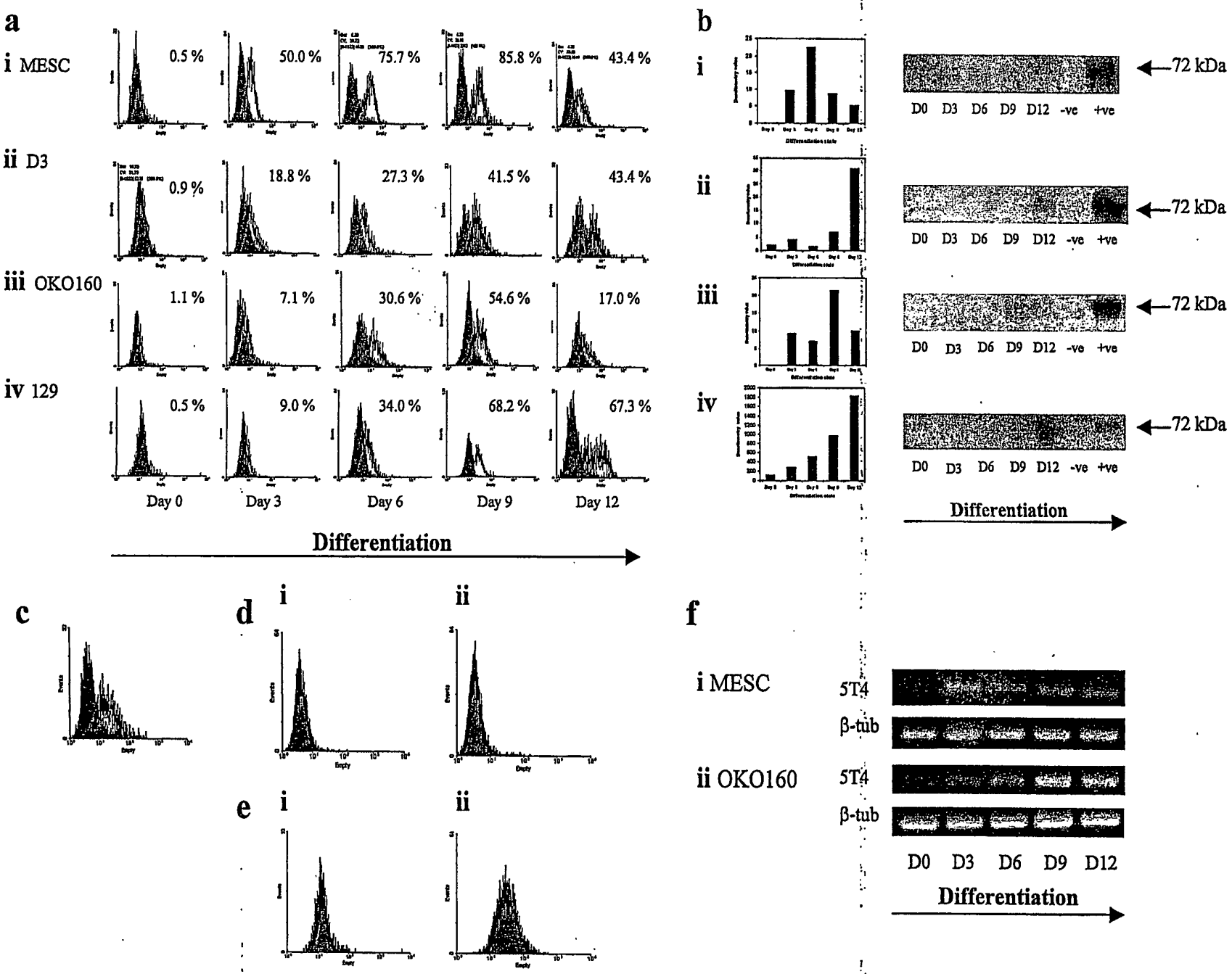

A
B
C
D
E
F

Figure 12


a
i MESC
0.5 %
50.0 %
75.7 %
85.8 %
43.4 %
ii D3
0.9 %
18.8 %
27.3 %
41.5 %
43.4 %
iii OKO160
1.1 %
7.1 %
30.6 %
54.6 %
17.0 %
iv 129
0.5 %
9.0 %
34.0 %
68.2 %
67.3 %
Day 0
Day 3
Day 6
Day 9
Day 12
Differentiation
b
i
ii
iii
iv
72 kDa
D0 D3 D6 D9 D12 -ve +ve
Differentiation
c
d
i
ii
e
i
ii
f
i MESC
5T4
β-tub
ii OKO160
5T4
β-tub
D0 D3 D6 D9 D12
Differentiation